February, 1954 *Price 35s. net* Vol. 10, No. 1

THE JOURNAL OF GENERAL MICROBIOLOGY

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CAMBRIDGE UNIVERSITY PRESS
LONDON: BENTLEY HOUSE, N.W.1
NEW YORK: 32 EAST 57TH STREET, 22

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JOURNAL OF GENERAL MICROBIOLOGY

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VOLUME 10, 1954

CAMBRIDGE
AT THE UNIVERSITY PRESS
1954

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)
and published by the Cambridge University Press
London: Bentley House, N.W. 1
American Branch: New York
Agents for Canada, India, and Pakistan: Macmillan

Kernforschungsanlage
Jülich
Zentralbibliothek

Pu 2770/ZB

(PC | Prof. Haul)

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No. 3, *Volume* 9 *of the Journal of General Microbiology was issued on* 10 *December* 1953.

Rose, R. E. & Miller, J. G. (1954). *J. gen. Microbiol.* **10**, 1–10.

Some Sampling Variations in Soil Fungal Numbers

By R. E. ROSE and JEAN G. MILLER

Rukuhia Soil Research Station, Hamilton, and Department of Agriculture, Wellington, New Zealand

SUMMARY: Fungal counts (as measured by direct plate counts) from soil samples taken from the same field have frequently shown variations of the order of 1 : 13 for some New Zealand pastures. The main source of error of mean counts is the variation between cores in the field, much less being due to subsampling and plating methods in the laboratory. For an arbitrary standard of accuracy, based on differences observed between soils at various stages of development, about twenty-five cores per field are necessary even if the field appears homogeneous. If the cores are to be bulked before plating thirty-two should be taken, preferably in eight groups of four cores. Little advantage accrues from taking more than three plates per sample for incubating and counting. Wide variations are occasionally obtained between batches on the mechanical shaker, but this could not be attributed to the effect of time of shaking.

In the course of some work to determine the average microfloral numbers of some New Zealand soils, large differences were found between counts from samples in the same field. The range of counts was considerably larger than that quoted by Waksman (1931), who found numbers varying from 8 to 25 millions per gram from fifty-one samples taken from a $\frac{1}{20}$ acre plot. This ratio of 1 : 3·25 is often exceeded on apparently uniform pasture, figures of 1 : 39 (0·2–7·9 millions) for actinomycetes, 1 : 32 (0·1–3·2 millions) for bacteria and 1 : 13 (50,000 to 641,000) for fungi being not uncommon from plots of $\frac{1}{40}$ acre of pasture. Consequently, Waksman's suggestion of five samples each of three or four borings, which has apparently been followed by other workers, seemed inadequate for New Zealand conditions. To determine the optimum number of samples for the purpose of future comparative studies, a series of trials was planned with soil samples from two different districts.

A. Rotorua district where the soil used for the trial was Taupo sandy silt, a moderately leached yellow brown pumice soil from Taupo ash.

This soil was further subdivided into three categories:

Virgin soil—an area of unused land of about $\frac{1}{4}$ acre in extent was used, the average distance between samples being about 10 ft.

New pasture—samples were taken from an area of about 1 acre in a valley from pasture about 2 years old.

Old pasture—a similar area to the above but the pasture was about 15 years old.

The vegetation on the virgin soil area was tussock, with some scrub. The pasture on the other two areas contained ryegrass, white clover and cocksfoot, with some to much Yorkshire fog, some crested dogstail, *Poa pratensis* and suckling clover.

B. Rukuhia Soil Research Station, where the soil is Hamilton clay loam, a moderately leached brown granular clay, derived from Hamilton (andesitic) ash.

The pasture sampled was dominantly perennial ryegrass—white clover, dense and evenly grazed, typical of a highly producing sward in the area.

METHODS

Procedure for collecting samples

A 1 in. cylindrical soil sampler was used throughout, the 0–3 in. layer being taken and removed and the sampler replaced to take the 3–6 in. layer, when this was required.

From each of the Rotorua soils twenty cores were taken in October 1950. These were each bagged separately, so that a measure of the variation between cores might be obtained. These samples were designated series 1. In June 1951, twenty cores were taken from each of the same areas and were bulked for each area. Samples were later drawn from these bulks, to determine subsampling variances, and these samples were called series 2. A repeat test of the bulk soil taken at this sampling time was made for the new pasture only (since the first tests showed some curious results) and results of this test were called series 3.

On the Hamilton soil, the first sampling aimed at establishing the relative variance due to location in the field, and due to the particular cores that were taken at any one site. Hence seven sites were chosen within an area of ¼ acre of gently undulating land, and at each site four cores were taken as closely together as possible, each layer of each core being bagged separately. These samples were called Hamilton series 1. On the same date, April 1951, on the same locality, two sets of twenty cores were taken and bulked, each layer being kept separate. This was to determine subsampling variances, and results from samples from these bulks were called Hamilton series 2, lots A and B. The cores comprising lot A were a short distance away from those comprising lot B, and were slightly farther down the slope.

Procedure for preparation of samples

For series 1 Rotorua soils each individual sample was sieved (2 mm. sieve) and 10 g. of soil taken for preparing the dilution. In many of the samples there was little more than 10 g. available from each core. For series 2 Rotorua soils the twenty cores yielded the following amount of sieved soil: virgin, 540 g.; new pasture, 205 g; old pasture, 392 g. After sieving and mixing, fifteen samples each of 10 g. were taken for the preparation of dilutions.

For series 1 Hamilton soil, individual samples were sieved and 10 g. taken, this representing about 20% of the sample.

For series 2 Hamilton soil each bulk was sieved and mixed and fifteen samples each of 10 g. were taken. Each sample in this instance represents about 1% of the total bulk.

Plating procedure

Dilutions for plating were prepared as follows: (*a*) 10 g. soil shaken with 100 ml. water = 1/10 dilution; (*b*) 5 ml. of (*a*) made up to 100 ml. and shaken = 1/200; (*c*) 1 ml. of (*b*) made up to 100 ml. and shaken = 1/20,000.

Each dilution was shaken for 5 min. on a Microid flask shaker.

One ml. of the final dilution was pipetted into a Petri plate and 10 ml. of medium held at 48° poured into the plate and evenly distributed. Standard methods of maintaining sterility were used throughout. Fungi were plated on a medium of the following composition, as used by Jensen (1934) in his investigations: glucose, 10·0 g.; asparagine, 1·0 g.; KH_2PO_4, 2·0 g.; $MgSO_4$, 0·5 g.; NaCl, 0·5 g.; agar, 25·0 g.; H_2O, 1000·0 ml.; adjusted to pH 4·6–4·8 and incubated for 4–5 days before counting.

In series 1 Rotorua soils four parallel plates were prepared from each dilution, for all other series three plates. In Rotorua old pasture, series 1, two plates were spoilt and could not be counted.

Procedure for counting

Direct counts were made of every colony, and for convenience statistical analyses have been carried out on these numbers. To give numbers of organisms per gram of soil, numbers per plate must be multiplied by the appropriate dilution figure, i.e. 20,000.

Distribution of counts

In studying the form of the distribution of the statistical population of fungal numbers as determined by the plate method, the appropriate variate is 'count per plate' of plates from the same soil sample. Although the different series of samples were taken to study different field sampling aspects, they can all be used to give estimates of the variance between plates prepared from the same sample. The estimates quoted for the variance in Table 1 are the values for the 'mean square within samples' from the analysis of variance tables for all soils. No transformation was applied to the variate, and all results are in units of the count per plate.

The agreement in the coefficients of variation for the two or three estimates for each type of soil is good, except possibly for the Rotorua old pasture. If the organisms are distributed among the plates according to a Poisson distribution, the plates variance of any series of samples should be equal to the corresponding mean plate count. A graph of the above values shows all points falling closely about the line 'variance = mean', with the exception of the Rotorua old pasture. A further test of the Poisson distribution hypothesis was made from the two series of samples of the Rotorua virgin soil. In series 1, when one aberrant core was omitted, the mean square between the nineteen remaining core means was less than the 'within cores' mean square, so that the seventy-six plates could be considered as belonging to one population. The χ^2 test for the fit of the data to a Poisson distribution gave $P = 0{\cdot}21$. The graph of the distribution shows that the deviations from the theoretical are

Table 1. *Means and variances between plates for all soils*

Origin of material		Mean count per plate	Degrees of freedom	Variance	Standard deviation	Coeff. of variation (%)
Rotorua, virgin soil	Series 1	7·1	60	9·9	3·2	45
	Series 2	5·9	30	8·1	2·8	47
Rotorua, new pasture	Series 1	13·9	60	10·5	3·3	24
	Series 2	39·4	30	46·8	6·8	17
	Series 3	14·5	16	9·1	3·0	21
Rotorua, old pasture	Series 1	33·1	58	64·0	8·0	24
	Series 2	24·2	30	15·2	3·9	16
Hamilton, 0–3 in.	Series 1	27·6	56	26·2	5·1	18
	Series 2, Lot A	24·9	30	17·6	4·2	17
	Series 2, Lot B	31·1	30	32·1	5·6	18
Hamilton, 3–6 in.	Series 1	16·7	56	16·9	4·1	25
	Series 2, Lot A	17·5	30	15·8	4·0	24
	Series 2, Lot B	24·0	30	27·5	5·2	22

erratic, and therefore it would be reasonable to expect a better fit if a larger sample were available. Series 2 of the Rotorua virgin soils, which consisted of three plates from each of fifteen subsamples from the one bulk of well-mixed soil was similarly tested and the Poisson distribution fitted with a χ^2 probability of 0·65.

Thus the tests agree that the counts per plate can be reasonably considered to show the characteristics of the Poisson distribution. As this is an expected distribution, the technique for plating and counting can therefore be considered satisfactory (Quenouille, 1948). The accuracy of the counts as descriptions of the number of organisms in the field can now be estimated, when the variances associated with each level of the sampling procedure have been ascertained.

Sampling stages and their variances

In obtaining the material for the plate counts three stages of sampling are normally used: (i) 'sites' are chosen in the field; (ii) at each site one or more cores are removed; (iii) from each core several plates are prepared. There is further sampling in the successive dilutions, but this has not been considered here, all operations being taken as part of the process.

Variance due to cores and sites

For all the samples except those in the Hamilton series 1, only one core was taken at each 'site' in the field. Hence the variance computed as 'between cores' includes the component of 'sites variance' also. One estimate of the two components separately is obtained later.

The estimates of this 'variance between cores' are shown in Table 2. For the Rotorua virgin soil, for which the fit of the Poisson was described above, all twenty cores were used in this estimation and the inclusion of the one discrepant core meant that the estimate of core variance component was positive, though small. The term standard deviation in that table is used to indicate the square root of the cores variance component. A graph of the above standard deviations (against the corresponding mean plate counts) shows them to lie approximately along a straight line, with no marked deviations. This indicates that although the number of degrees of freedom available for the estimation is in each case rather small, there is no evidence that any estimate is a 'freak' one which should not be used in further calculations.

Table 2. *Estimates of components of cores variance*

Origin of material	Cores variance component	Degrees of freedom	Mean	Standard deviation
Rotorua virgin soil series 1	4·0	19	7·1	2·0
Rotorua new pasture series 1	27·0	19	13·9	5·3
Rotorua old pasture series 1	243·0	19	33·1	15·6
Hamilton, 0–3 in. series 1	215·0	27	27·6	14·8
Hamilton, 3–6 in. series 1	88·0	27	16·7	9·4

Comparison of the cores variances in Table 2 with the plates variances in Table 1 shows that for each case except Rotorua virgin soil the cores variance is much higher than the plates variance. Now, one of the limiting factors to the amount of microbiological investigation that can be undertaken by a laboratory is the number of plates to be counted at one time. It is also desirable to keep the number of plates as small as possible on account of the amount of labour and time required for their preparation and the limitations in space for incubation. Consequently, a sampling scheme must be chosen to give the greatest amount of information from the fewest number of plates. In view then of the relative sizes of the 'plates' and 'cores' variance in general, the most efficient sampling scheme will use the fewest number of plates per core. Because accidents are liable to happen to the plates, such as complete overgrowth by a different organism which prohibits counting, the minimum practical number would be three per core. The variance of the mean counts of different cores would then be: $\text{var.}_C + \frac{1}{3}\text{var.}_P$, where var._C is cores variance, var._P is plates variance, and the S.E. per core is the square root of this variance.

Since the distribution of plate counts has been established as a Poisson, the best estimate for the var._P for each soil is the mean for that soil. Using these values, the standard errors per core are shown in Table 3 for this series of soils in units of one plate.

Table 3. *Estimates of standard errors of mean counts per core, from three parallel plates for each*

Soil	Variance per core	Standard error per core
Rotorua virgin soil	6·2	2·6
Rotorua new pasture	31·7	5·7
Rotorua old pasture	255·0	16·0
Hamilton, 0–3 in.	224·0	15·0
Hamilton, 3–6 in.	93·6	9·7

From these standard errors can be calculated the number of cores required for any given degree of accuracy. No standard of accuracy can be finally chosen until there is some knowledge of the variations in mean counts which could be expected in the same soil between different sampling times when no progressive change has been taking place. A knowledge of the difference in fungal counts as related to differences in production level is also necessary. An arbitrary decision was made on the differences that would be required to be measured in future work, for each of the soil conditions. The number of cores is then calculated so that, if the variance remains the same as in the present investigation, then it is expected that any measured differences which are as great as those specified differences will be judged statistically significant. The formula for calculating the required number of cores must then be

$$r = \frac{2 \times \text{variance per core}}{(\text{required difference})^2} \times t^2$$

where t is the usual tabulated 'Student's t', for the probability level at which it is desired to test the hypotheses under investigation. The results of this calculation for all the soils studied, for values of t corresponding to the 5% probability level are shown in Table 4. These indicate that in the present state of knowledge a working standard of twenty to twenty-five cores should be adopted.

Table 4. *Numbers of cores required for the adopted standards of accuracy*

Soil	Mean count	Difference to be measured	Number of cores required
Rotorua virgin	7·1	2	15
Rotorua new pasture	13·9	4	17
Rotorua old pasture	33·1	10	22
Hamilton, 0–3 in.	27·6	10	31
Hamilton, 3–6 in.	16·7	6	23

Location of sampling positions in the field

The above results were obtained from single cores taken at random over the field. To test the effect of taking larger cores, or of group sampling, one trial was conducted at Hamilton (series 1), where four almost contiguous cores were taken at each 'site' in the field. Samples were taken at two depths and from results of these the components of variance of the sites in the field and of the cores were estimated. The taking of larger cores will not necessarily give the same results as cluster sampling. However, in this trial the cores were taken so closely together that they may almost be considered to form one large core.

	Variance due to cores	Variance due to sites	Mean count per core
0–3 in. layer	284	54	27·6
3–6 in. layer	36	52	16·7

Apparently for the 3–6 in. layer considerable accuracy will be lost by taking several cores at one site, since the sites variance is larger than that due to cores. For the 0–3 in. layer, however, the sites variance contributes only a small amount to the total, and accordingly much inconvenience may be avoided, with little loss of accuracy by taking the cores in clusters. However, this one trial does not supply sufficient consistent evidence for cluster sampling to be recommended.

Bulking of samples before plating

For most of this technique investigation, the total amount of soil in each core was used in making the first dilution and its identity kept separate. For routine testing, however, it is envisaged that the required number of samples will be taken from the field then bulked in groups, and one sample will be drawn from each bulk for plating. Only a few degrees of freedom will then be available for estimating the error variance, but if this proves to be in agreement with expectation, as indicated by these individually plated samples, the mean count can be credited with a standard error based on the number of cores taken and not on the small number of subsamples actually planted. The means will then, however, have another component of error, due to subsampling from the bulk, as only a fraction of the soil will be used in making up a dilution. In order to estimate this component, twenty cores were taken from each of the soils. These were bulked and the bulks were thoroughly sieved and mixed. From each bulk fifteen subsamples were drawn and three plates made from each subsample.

Table 5. *Components of sub-sampling variance*

Soils	Variance comp. of subsampling	Variance of plates	Mean count
Rotorua virgin series 2	0·0	8·1	5·9
Rotorua new pasture series 2	492·0	46·8	39·4
Rotorua new pasture series 3	3·1	9·1	14·5
Rotorua old pasture series 2	7·5	15·2	24·2
Hamilton, 0–3 in. series 2, lot A	7·2	32·1	24·8
Hamilton, 0–3 in. series 2, lot B	21·7	16·9	31·1
Hamilton, 3–6 in. series 2, lot A	8·7	15·8	17·5
Hamilton, 3–6 in. series 2, lot B	9·8	27·5	24·0

As the bulk of soils from which the samples are drawn is a finite amount, not infinite as in a field, the error per subsample, due to the subsampling process, is given by $\sqrt{[\text{var.}_S\,(1-f)]}$, where var._S is the subsampling variance and f is the fraction of the total bulk taken in each sample.

Thus the full error variance of a mean count derived from n cores which are bulked into q groups from each of which one sample is taken to give r plates, is

$$\frac{\text{var.}_C}{n}+\frac{\text{var.}_S\,(1-f)}{q}+\frac{\text{var.}_P}{qr}, \tag{1}$$

where var._C is variance component of cores, var._S is variance component of subsampling, var._P is variance component of plates.

The values of the expected standard error per mean count from the soils under consideration were then calculated under the assumption of a sample of twenty-five cores being taken, these cores then being grouped into five bulks from each of which three plates would be prepared. From measurements of the soil volume weights the following values of 'f', the fraction of the bulk which would be taken in the sample, were found to be:

Rotorua virgin soil	$f=0\cdot07$	$1-f=0\cdot93$
Rotorua new pasture	$f=0\cdot20$	$1-f=0\cdot80$
Rotorua old pasture	$f=0\cdot10$	$1-f=0\cdot90$
Hamilton 3–6 in. (A & B)	$f=0\cdot01$	$1-f=0\cdot99$

Then the estimations for the standard errors would be as follows:

	Contributions to variance		
Soil	Cores	Subsampling	Plates
Rotorua virgin	$\frac{4\cdot0}{25}$ +	$\frac{0\cdot93\times0\cdot0}{5}$ +	$\frac{8\cdot1}{15}=0\cdot16+0\cdot0+0\cdot54=0\cdot70$
New pasture	$\frac{27\cdot1}{25}$ +	$\frac{3\cdot1\times0\cdot80}{5}$ +	$\frac{9\cdot1}{15}=1\cdot08+0\cdot49+0\cdot61=2\cdot18$
Old pasture	$\frac{243\cdot0}{25}$ +	$\frac{7\cdot5\times0\cdot90}{5}$ +	$\frac{15\cdot2}{15}=9\cdot72+1\cdot35+1\cdot00=12\cdot07$
Hamilton (A and B)	$\frac{88\cdot0}{25}$ +	$\frac{9\cdot0\times0\cdot99}{5}$ +	$\frac{20\cdot0}{5}=3\cdot52+1\cdot80+1\cdot83=6\cdot65$

The subsampling from bulks has added appreciably to the error in most cases, and it would appear to be worth while to increase the number of bulks. This will have an effect on both the second and third components as they contain the number q in the denominator, so that an effective decrease could be obtained by increasing q to about 8. That is, a sampling scheme of twenty-four cores grouped into eight bulks would be adequate, while an increase to thirty-two cores grouped in fours would be more likely to give the required standard of accuracy with little increase in the work.

If this bulk method of testing is adopted, then in some circumstances the estimates of the variance components found in this paper can be used to give more accurate estimates of the standard errors of the mean counts than can be obtained from use of the results in the new test.

Thus, if thirty-two cores are taken, and are bulked in lots of four from each of which one subsample is drawn and three plates made, a set of eight mean counts, one per bulk will be obtained. If these are $x_1 \ldots x_8$, the mean square between bulk means will be, in units of one plate count $\frac{3}{7}\Sigma(x_i-\bar{x})^2=A$ and the standard error of the field mean count would be calculated $\sqrt{(\frac{1}{24}A)}$.

However, if it can be shown that the variances are of the same order as those in this present investigation then the variance estimates, shown in these tables, being based on more degrees of freedom, will give a better estimate of the total standard error. The mean square A has expectation $\text{var.}_P+3\,\text{var.}_S+3\,\text{var.}_B$ (where var._P and var._S are as before and var._B is the variance due to bulks), and since each bulk consists of four cores taken at random $\text{var.}_B=\frac{1}{4}\,\text{var.}_C$. Now the mean square between plates has expectation var._P and hence (mean square between bulks (i.e. A) $-$ mean square between plates) has expectation $3\,\text{var.}_S+\frac{3}{4}\,\text{var.}_C$. Now if this quantity as calculated from the values in

Tables 2–5 agrees with the observed difference of the mean squares, it can be assumed that the same conditions exist at the 2 times of sampling, and hence the tabled values can be used for insertion in the formula (1), which then becomes

$$\text{S.E. of the general mean} = \sqrt{\left(\tfrac{1}{32}\text{var.}_C + \tfrac{1}{8}\text{var.}_S + \tfrac{1}{24}\text{var.}_P\right)}$$

The factor $(1-f)$, for sampling from a finite population must of course be used with the subsampling variance throughout the above calculations wherever the amount taken in the subsample becomes an appreciable amount of the bulk.

Preparation of plates

When estimates of the subsampling variance were being obtained it was noticed that the subsampling mean square for Rotorua new pasture was very large (being 492). An examination of the counts showed a clear subdivision into groups of four samples which coincided exactly with the groups which were put on the mechanical shaker. When the variance due to 'shakings' was removed the mean square between samples was redued to 6·3. The only other time when this effect could be detected was in the Rotorua old pasture, where it was slight. A repeat test was made from the same bulk of Rotorua new pasture soil, and this, shown as series 3, did not show the effect at all, besides giving a value for the mean count more in line with series 1. This test was taken as the correct one, and was used in estimating the error due to subsampling for this soil.

An investigation was made into the effects of different shaking times. A set of sixteen samples (from the same soil) was prepared and was put on to the shaker in subsets of four, and given widely different shaking times. Results are shown in Table 6.

Table 6. *Mean counts for different shaking times*

Shaking time (min.)	Mean count per plate
$2\frac{1}{2}$	9·7
5	14·4
10	16·4
20	13·2

The differences just fail to reach significance level when tested against the variance of individual sample means, and are certainly not of the order which was encountered in the suspect Rotorua new pasture series. The sample means, however, showed significant differences among themselves when tested against the plates variance. A more complete investigation of this point may be undertaken later.

DISCUSSION

The sampling scheme which has been proposed for New Zealand soils will measure with accuracy differences in fungal numbers such as have been shown to exist here between undeveloped lands and high-producing pastures. The variability in the higher producing pastures is greater than that of virgin soils and apparently of arable land, and this may be due to the effects of the grazing animals. However, it will still be possible with the facilities of a small laboratory to obtain accurate mean values for fields under study provided sufficient field samples are taken.

The mean values of counts in Table 1 for the same soils at different samplings give an indication that seasonal and even diurnal changes may be relatively large, and sampling schemes may have to consider this factor also. The ultimate aim of these laboratory investigations is to trace the changes in soil microflora associated with treatments which change the production level. Differences in physiological groups and types of organisms are as important as changes in numbers, but a sample which gives a satisfactory estimate of numbers is necessary before changes in kind can be studied.

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(*Received* 1 *April* 1953)

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Studies on the Antibacterial Action of Spermine

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SUMMARY: Spermine was found to inhibit the growth of various micro-organisms. The most susceptible were *Staphylococcus aureus* and *Staph. albus*, *Neisseria gonorrhoeae* and *N. meningitidis*, and to a lesser degree *Bacillus anthracis*. The Enterobacteriaceae were relatively resistant. The action of spermine was enhanced by an increase in pH of the medium. The antibacterial effect was bactericidal; it was slowed but not prevented by a decrease in temperature from 37 to 6°. The nutrients of various media influenced the action of spermine.

Spermine, a biological polyamine, is a substance of controversial nature. It has been shown to possess growth-inhibiting properties against various micro-organisms (Bichowsky-Slomnitzki, 1948; Gurevitch, Rozansky, Weber, Brzezinsky & Eckerling 1951; Hirsch & Dubos, 1952; Rozansky, Gurevitch, Brzezinsky & Eckerling, 1949) and growth-promoting activity for *Haemophilus parainfluenzae* (Herbst & Snell, 1948). It has also been found to counteract the antibacterial effect of atebrine, propamidine and quinine on *Escherichia coli* (Silverman & Evans, 1943, 1944; Miller & Peters, 1945). Spermine is widespread in human and animal body tissue (Guggenheim, 1940; Harrison, 1933). Its role, if any, as an antibacterial agent which affords natural resistance to the body, is unknown. Further studies of this material therefore seemed worthwhile. The present investigation is concerned with the following aspects of the antibacterial action of spermine: mode of action; influence of pH; influence of temperature; sensitivity of different bacteria.

MATERIALS AND METHODS

Spermine tetrahydrochloride (Hoffmann La Roche) was used throughout the experiments. The micro-organism studied most extensively was *Staphylococcus aureus* no. 23, a haemolytic coagulase and mannitol positive strain. The other bacteria used are listed in Table 2.

Antibacterial assays of spermine were made by (*a*) serial dilution and (*b*) the cup method. Serial dilutions of spermine were made in Wassermann tubes (13 × 100 mm.) containing 2 ml. of Difco nutrient broth. Controls without spermine were included in all experiments. The cup method was the same as previously reported (Rozansky *et al.* 1949). Difco nutrient agar was used except when otherwise specified.

RESULTS

Influence of pH *value of the medium on the growth inhibition of* Staph. aureus *by spermine*

The antibacterial activity of spermine rose gradually with the increase of the pH of the medium (see Fig. 1). Change in pH value from 6·2 to 8·75 decreased the amount of spermine required to obtain complete inhibition of *Staph. aureus* from 1000 µg./ml. to 2 µg./ml. The growth of the test organism in the control tubes, i.e. in the absence of spermine, was almost unaffected by these changes in pH value.

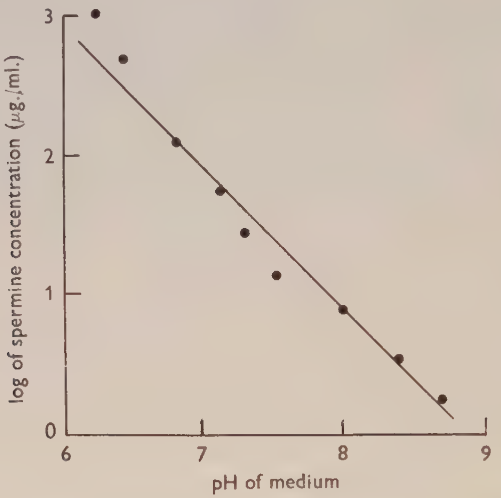

Fig. 1. Minimal concentration of spermine inhibiting the growth of *Staph. aureus* at various pH values of the medium. Size of inoculum *c.* 10^4 cells/ml. Medium: Difco nutrient broth. Results read after 48 hr. incubation. Inhibition i.e. no visible growth.

Bactericidal effect of spermine

In experiments with *Staph. aureus* 23, the size of inoculum did not affect appreciably the inhibitory activity of spermine. A 100- and 10,000-fold increase of the inoculum required a twofold and eightfold increase of spermine respectively.

These results suggested that the drug exerted a bactericidal effect. In order to test this, *Staph. aureus* was inoculated into flasks of Difco nutrient broth (pH 7·7) containing 25 µg./ml. and 50 µg./ml. of spermine and into control flasks without spermine. The flasks were incubated at 37° and at varying intervals of time (Table 1) samples were taken for the determination of viable counts. The pour-plate method was used, and plates were incubated for 48 hr. at 37°. All counts were performed in triplicate.

At 37° (see Table 1) 25 µg. spermine/ml. for 3 hr. killed 70 % of the staphylococci. Fifty µg. spermine/ml. were even more effective, killing more than 99 % of the bacteria in 3 hr. After 10 hr. incubation all flasks containing 25 and 50 µg. spermine/ml. were sterile.

In order to determine whether spermine was effective against non-multiplying bacteria, broth samples containing varying concentrations of spermine were inoculated with *Staph. aureus* 23. The flasks were kept at 6° and the number of surviving bacteria was determined after 24, 72 and 96 hr. by the plate-count technique. Controls without spermine were run simultaneously.

Table 1. *Effect of spermine on* Staphylococcus aureus *in Difco nutrient broth*, pH 7·7

		Concentration of spermine (μg./ml.)								
		0	25		50		100		400	
Temp. of contact (°)	Time of contact (hr.)	No. of colonies in thousands*	No. of colonies in thousands*	% of survivors	No. of colonies in thousands*	% of survivors	No. of colonies in thousands*	% of survivors	No. of colonies in thousands*	% of survivors
6	0	14	13	100	12	100	13	100	14	100
	24	14	3·3	25·3	0·46	3·8	0·18	1·4	0·03	0·2
	72	8	0·3	2·3	0·05	0·4	0·015	0·1	0	0
	96	7	0·08	0·6	0·015	0·13	0·015	0·1	0	0
37	0	14	8	100	10	100	—	—	—	—
	3	18	2·3	30	0·06	0·6	—	—	—	—
	10	12,000	0·04	0·5	0	0	—	—	—	—
	24	900,000	0	0	0	0	—	—	—	—

* Number of colonies developing on agar plates poured with 1 ml. samples of the broth. Counts performed after 48 hr. incubation of the plates at 37°.

It is seen from Table 1 that spermine killed the staphylococci in the cold. The action at 6° was however markedly slower than at 37°, 25 μg./ml. killed about 75 % of the staphylococci in 24 hr.; 50 μg./ml. killed 96 % of the cells in the same time; 400 μg./ml. killed 99·8 % after 24 hr.

Susceptibility of different micro-organisms to spermine

Table 2 shows that the organisms most sensitive to the inhibitory action of spermine were staphylococci, both aureus and albus types. In Difco nutrient agar at pH 7·3 and 7·5, 50 and 25 μg. of spermine, respectively, diluted in 0·1 ml. of saline were required to produce a clear zone of inhibition (not less than 12 mm. in diameter) by the cup technique. In Difco nutrient broth at the same pH values (i.e. 7·3 and 7·5) a concentration of 30 and 15 μg./ml. caused full inhibition.

Neisseria gonorrhoeae and *N. meningitidis* tested on glucose starch agar (Difco) and ascites agar were inhibited to a similar extent to that found with staphylococci.

Various Gram-negative rods of the family Enterobacteriaceae were only slightly affected by the drug. *Shigella flexneri*, however, was found to be quite sensitive at an alkaline pH. Haemolytic streptococci and *Corynebacterium hofmanni* were tested for their sensitivity to spermine in nutrient broth enriched with brain heart infusion (4 parts Difco nutrient broth and 1 part Difco brain heart infusion broth) and were found to be inhibited by 250 μg./spermine ml. (Table 2).

COMMENT

The degree of inhibition of bacterial growth by spermine depended on several factors. One of these was the pH of the medium. In Fig. 1 an almost straight line was obtained when the inhibitory activity of spermine on *Staph. aureus* 23 was plotted against the pH of the medium. It seemed, therefore, that the undissociated molecule rather than the ionized form was responsible for the antibacterial properties. The influence of the pH was less marked with *Bacillus anthracis* and *Sh. flexneri* and still less evident with the more resistant enterobacteriaceae and *Streptococcus faecalis* (Table 2). Hirsch & Dubos (1952) found that the inhibition of growth of *Mycobacterium tuberculosis*, an organism highly susceptible to spermine, was independent of the pH value of the medium between 6·3 and 7·4.

The action of spermine was primarily bactericidal and was also evident against non-multiplying organisms. Hirsch & Dubos (1952) have demonstrated the bactericidal effect of spermine for *Myco. tuberculosis*.

The activity of spermine was influenced by the medium employed. Organisms grown in brain-heart infusion were much less affected than in nutrient broth (Table 2). The spermine antagonizing effect of Bacto stock agar has been described previously (Gurevitch *et al.* 1951). It is possible that the relatively high resistance of haemolytic streptococci and *C. hoffmani* which were tested in brain-heart infusion was due to the presence of spermine antagonists in these complex media.

Table 2. *Inhibition of growth of various micro-organisms by spermine*

			Concentration of spermine showing inhibition using		
Strain	Medium	pH of medium	Dilution method μg./ml.*	Cup method μg./0·1 ml.†	Remarks
Staph. aureus 23	Nutrient broth or agar	6·2	1000	—	—
	Nutrient broth or agar	6·4	500	—	—
	Nutrient broth or agar	6·8	125	100	—
	Nutrient broth or agar	7·1	60	—	—
	Nutrient broth or agar	7·3	30	50	—
	Nutrient broth or agar	7·5	15	25	—
	Nutrient broth or agar	8·0	8	12	—
	Nutrient broth or agar	8·4	6	6	—
	Nutrient broth or agar	8·7	2	—	—
	4 parts nutr. br. and 1 part brain-heart broth	7·1	250	—	—
	4 parts nutr. br. and 1 part brain-heart broth	7·9	60	—	—
Staph. albus B	Nutrient broth or agar	6·8	500	—	—
	Nutrient broth or agar	8·0	4	—	—
Staph. albus H5	Nutrient broth or agar	6·8	30	—	—
	Nutrient broth or agar	8·0	1	—	—
B. anthracis	Nutrient broth or agar	6·2	1000	—	—
	Nutrient broth or agar	6·8	500	100	—
	Nutrient broth or agar	7·3	250	—	—
	Nutrient broth or agar	7·9	125	—	—
	Nutrient broth or agar	8·7	60	25	—
Sh. flexneri 1150	Nutrient broth or agar	6·4	1000	—	—
	Nutrient broth or agar	7·0	250	—	—
	Nutrient broth or agar	8·0	125	50	—
	Nutrient broth or agar	8·5	125	—	—
	Nutrient broth or agar	8·8	—	25	—
E. coli 055	Nutrient broth or agar	6·8	1000	—	—
	Nutrient broth or agar	8·0	250	—	—
Sal. typhi	Nutrient broth or agar	6·8	1000	—	—
	Nutrient broth or agar	7·3	1000	—	—
Str. faecalis	Nutrient broth or agar	6·8	1000	—	—
	Nutrient broth or agar	7·3	1000	—	—
Str. faecalis 19	Nutrient broth or agar	6·8	1000	—	—
	Nutrient broth or agar	8·0	1000	—	—
Myco. phlei	Nutrient broth or agar	6·8	1000	—	—
	Nutrient broth or agar	7·7	1000	—	—
C. hofmannii	4 parts nutr. br. and 1 part brain-heart broth	7·1	250	—	—
Str. haemolyticus	4 parts nutr. br. and 1 part brain-heart broth	7·1	250	—	2 strains
N. gonorrhoeae	Dextrin starch agar	7·5	—	50	5 strains
	Ascites agar	7·6	—	50	2 strains
N. meningitidis	Dextrin starch agar	7·5	—	50	4 strains
	Ascites agar	7·6	—	50	2 strains

* Concentration of spermine causing inhibition of visible growth after 48 hr. incubation at 37°. Inoculum *c.* 10,000 organisms/ml. Twofold dilution technique.

† Concentration of spermine causing a clear inhibition zone of not less than 12 mm. in diameter, 0·1 ml. of samples containing the specified amounts were introduced into the cups.

The authors wish to thank Mrs R. Kushnir-Yeivin, M.Sc., for assistance in some of the experiments. The tests with Neisseria were performed by one of us (R.R.) at the Statens Seruminstitut in Copenhagen. Appreciation is expressed to Dr J. Ørskov, Director of the Institute, and to Dr A. Reyn, Chief of the serodiagnostic department, for facilities provided, and to Dr J. Bang for valuable advice and assistance.

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(*Received* 29 *April* 1953)

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The Mineral Nutritional Requirements of *Mucor hiemalis* Wehm.

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SUMMARY: A series of four factorial experiments with culture media was carried out dealing with (1) the action of individual salts and their interactions in the medium, (2) the relation of the nitrogen source to the action of the salts, (3) the effect of the ratio of the carbon source and (4) the proportionate influence of the salts in the medium. Statistical analysis of the results has shown (*a*) the composition of the culture medium which produces optimal growth under given conditions, (*b*) that a suitable balance of salts in the medium, while conducive to good growth, is not of over-riding importance to this fungus, and (*c*) that ammonium nitrate serves as the key nutrient in the balance of salts which produces optimal growth measured as milligrams of dry weight.

The real study of the nutrition of fungi probably began when Raulin introduced his defined medium in 1869. Since that time there have been numerous accounts of, and references to, fungal nutrition, but there is a lack of statistical investigations of the food requirements of fungi which are complete from one aspect of nutrition. Such investigations, which of necessity cover a limited field, give a reliable picture of the behaviour or response of the fungi concerned expressed in some arbitrary but standard terms such as dry weight. The results are of value because they can serve as the basis for systematic comparisons under fixed and reproducible conditions of growth and for metabolic investigations which are becoming of increasing importance in the study of fungal physiology, and they also have an industrial application. As early as 1922, Young & Bennett, using *Macrosporium sarcineforme*, *Fusarium oxysporum* and *Phoma apicola* with the salts calcium nitrate, acid potassium phosphate and magnesium sulphate at different molar concentrations, found that a correct balance of the mineral elements was essential for maximum growth, but that this behaviour varied according to the species of fungus used as test organism. Other workers have also obtained results indicative of a similar conclusion, until Talley & Blank (1941) gave the results of their careful statistical analysis of the nutritional requirements of *Phymatotrichum omnivorum*, the causal organism of the root rot disease of cotton and other plants. These results showed that the absolute amount of certain essential metabolites was not so important as the balance between them. While the balance of salts may affect the overall metabolism of the fungus there are also indications that it may affect the synthesis of individual compounds such as penicillin. For instance, Pratt (1945) found that variations in the concentrations of KH_2PO_4,

$MgSO_4$ and $NaNO_3$ affected the amount of penicillin produced by *Penicillium notatum* in surface cultures. It seems apparent, therefore, that the proportion of salts in a defined medium must be taken into account in order to obtain good growth of fungi. Moulds are now being used side by side with green plants as material for fundamental investigations in plant metabolism, and thus the nutritional requirements of the fungi used take on an added importance and become the starting point for some of these investigations. In this paper a critical study of the mineral food requirements of complementary strains of *Mucor hiemalis* is reported. No previous work on the nutrition of this fungus seems to have been done. The statistical work has been modelled on that of Talley & Blank (1941) because their method simplifies interpretation of the results of the experiments.

MATERIAL AND METHODS

The complementary strains of *M. hiemalis* Wehm. used in this investigation were obtained from the Commonwealth Mycological Institute, Kew, cultures nos. 21216, 52 and 21217, 53. For general growth of stock cultures Blakeslee's agar was used (malt extract 20 g., glucose 20 g., peptone 1 g. and agar 20 g., made up to a litre with water). For preliminary experiments the following basal defined liquid medium was used: glucose 20 g., $MgSO_4.7H_2O$ 0·5 g., K_2HPO_4 1·0 g., KCl 0·5 g., $FeSO_4$ 0·001 g., NH_4NO_3 1·6 g., and water to 1 litre. In all experiments the K_2HPO_4 was added aseptically and separately, after autoclaving, to the other constituents of the media. The inoculum for liquid cultures was prepared from fully mature sporing cultures on agar which were shaken with 10 ml. of sterile distilled water. Approximately 1 ml. of the resulting spore suspension was then used to inoculate each culture flask. It was found by trial that the exact amount of inoculum, provided it did not contain a very small number of spores, did not significantly affect the final dry weight.

Preliminary experiments showed that the optimum temperature for mycelial growth was 25°. The mycelial yield was also high at 20°, and this was the temperature used in these experiments. Although there was a slight difference in the rate of growth of the plus and minus strains consistent results were obtained in preliminary work when both strains were grown for 5 days. Analar chemicals were used throughout the work; the addition of 2 p.p.m. of Zn, Cu, Co, Mn and Mb did not affect the results.

RESULTS

Factorial experiments were designed to study the concentration of the nutrient salts which might influence the results, and to assess the response brought about by the interaction of concentrations of two or three salts. The basis of the work is that the fungus is grown in all possible combinations of the salt concentrations chosen for study.

First factorial analysis

The first factorial experiment concerned the salts KCl, K_2HPO_4 and $MgSO_4.7H_2O$. The KCl was present either at 0·002 M or not added; it is always present in minute quantities in the chemicals used. The K_2HPO_4 was used at molar concentrations of 0·004, 0·008 and 0·012, while the $MgSO_4.7H_2O$ was at molar concentrations of 0·0015, 0·003 and 0·006. Thus there were eighteen possible combinations, and with five replicates each, making a total of ninety cultures in any one experiment. All cultures were grown for 5 days, the mycelial mat was filtered off, dried carefully just below 100° to prevent charring, and weighed. All results were expressed as milligrams of dry weight.

The results of the first factorial experiment are given in Table 1, and the analysis of variance is contained in Table 2. In this analysis odds of 99 : 1 are

Table 1. *Growth responses of* Mucor hiemalis (+) *and* (−) *strains, to varying concentrations of* K_2HPO_4, $MgSO_4$ *and* KCl

Results given as mg. dry weight

	Mean weight			Mean weight	
K_2HPO_4	+	−	$MgSO_4$	+	−
0·004 M	34·98	34·81	0·0015 M	39·42	36·81
0·008 M	32·83	33·78	0·003 M	35·24	39·14
0·012 M	47·30	45·97	0·006 M	40·46	38·64

	$MgSO_4$					
	0·0015 M Mean weight		0·003 M Mean weight		0·006 M Mean weight	
K_2HPO_4	+	−	+	−	+	−
0·004 M	36·76	31·51	30·87	32·69	37·33	40·23
0·008 M	28·39	30·23	30·86	36·37	39·26	34·74
0·012 M	53·12	48·63	43·99	48·37	44·80	40·86

	KCl, none Mean weight		KCl, 0·002 M Mean weight	
	+	−	+	−
	36·74	36·23	40·07	40·14
$MgSO_4$				
0·0015 M	36·86	34·46	40·15	39·14
0·003 M	33·43	40·68	37·05	37·60
0·006 M	38·12	33·53	42·8	43·68
K_2HPO_4				
0·004 M	33·10	32·38	38·86	37·24
0·008 M	29·86	31·86	35·80	35·69
0·012 M	47·26	44·44	47·67	45·70

	$MgSO_4$											
	0·0015 M Mean weight				0·003 M Mean weight				0·006 M Mean weight			
KCl	0		0·002 M		0		0·002 M		0		0·002 M	
	+	−	+	−	+	−	+	−	+	−	+	−
K_2HPO_4												
0·004 M	37·72	32·96	35·8	30·06	32·08	32·42	29·66	34·96	29·52	33·76	45·14	48·7
0·008 M	24·78	26·48	31·96	27·98	23·48	38·84	38·24	33·9	41·32	30·46	37·2	39·2
0·012 M	53·56	43·96	52·68	53·40	44·72	52·8	42·86	51·94	44·4	36·56	46·08	45·16

highly significant, while odds of 19 : 1 are taken as only being indicative of significance. Detailed consideration of these results is given below.

Magnesium sulphate; the plus and minus strains responded differently. The *F* value for the (+) strain indicates that the results are significant, while those for the (−) strain are not.

Dipotassium hydrogen phosphate; with both (+) and (−) strains the results are significant. An increase of 12·3 mg. was obtained by increasing the molarity of K_2HPO_4 from 0·004 to 0·012 for the (+) strain and a similar increase was seen for the (−) strain, which indicates that this molarity is favourable for this fungus.

Potassium chloride; the results for both strains agree closely, but the difference between the presence or absence of this salt is only of slight significance. A difference of only 4 mg. exists between the mean dry weights of (+) and (−) strains produced when this salt is present or absent. Chloride ions are therefore not required in these cultures or sufficient of the element may be present as impurity in the chemicals used.

Table 2. *Analysis of variance, Exp.* 1

			F		
				Required odds	
(+) strain	D.F.	Mean square	Found	99 : 1	19 : 1
Total	89	115·14	—	—	—
$MgSO_4$	2	229·33	3·806	4·92	3·13
K_2HPO_4	2	1828·04	30·43	4·92	3·13
KCl	1	238·1	3·968	7·01	3·98
$MgSO_4 \times KCl$	2	6·61	—	—	—
$K_2HPO_4 \times KCl$	2	65·7	1·09	4·92	3·13
$K_2HPO_4 \times MgSO_4$	4	239·34	3·97	3·6	2·5
Error	76	60·25	—	—	—
(−) strain					
Total	89	105·88	—	—	—
$MgSO_4$	2	—	—	—	—
K_2HPO_4	2	1371·02	16·48	4·92	3·13
KCl	1	344·96	4·15	7·01	3·98
$MgSO_4 \times KCl$	2	—	—	—	—
$K_2HPO_4 \times KCl$	2	6·12	—	—	—
$K_2HPO_4 \times MgSO_4$	4	—	—	—	—
Error	76	83·21	—	—	—

Interaction of salts of the first analysis

This experiment also shows the interaction of the first and second order of salts, i.e. the salts taken in the three possible pairs and all together. The results of the first and second order interactions are also given in Tables 1 and 2.

There is a divergence of response between the two complementary strains of Mucor regarding the interaction of $MgSO_4.7H_2O$ and K_2HPO_4. With the (−) strain the results show no significance, but with the (+) strain there is significance. The response of *M. hiemalis* (+) strain is most marked when the $MgSO_4.7H_2O$ is at the lowest concentration of 0·0015 M. The average dry weight then produced was 53·12 mg., which was 8·32 mg. greater than the next highest weight. The lower level of $MgSO_4.7H_2O$ did not influence the response of 0·005 M- and 0·008 M-K_2HPO_4, but it did affect the response to 0·012 M-K_2HPO_4. At all levels an increase of growth results with increase of phosphate concentration to the highest level of 0·012 M. The conclusion to be drawn is that it is a special balance between these two salts which is important in increasing the amount of growth. In their experiments with *Phymatotrichum omnivorum*, Talley & Blank (1941) also found that the correct physiological balance between K_2HPO_4 and $MgSO_4.7H_2O$ was a deciding factor in increasing growth of the fungus. In their case growth increased as the concentration of both salts increased, whereas with *M. hiemalis* (+) strain growth increased with the lowest level of $MgSO_4.7H_2O$ combined with the highest level of K_2HPO_4.

The interaction of K_2HPO_4 and KCl is not significant, but an increased growth is shown for both (+) and (−) strains when KCl was added at the lower levels of 0·004 M- and 0·008 M-K_2HPO_4. The single salt analysis indicated that the action of the chloride ion in growth is doubtful when added as KCl with potassium already present. Hence the result of the first order interaction of KCl and K_2HPO_4 probably indicates that at the lower levels of K_2HPO_4, potassium becomes a limiting factor, and that the deficiency is made up by addition of KCl.

With the (+) strain the interaction of $MgSO_4.7H_2O$ and KCl was not significant, while with the (−) strain the results indicate no significance for the interaction of any of the salts used. As regards the third order of interaction, i.e. between the three salts taken together, the results above indicate that this interaction is not significant since only one of the first order interactions proved to be so.

Second factorial analysis

It has been noted by various workers that the source of the nitrogen available to fungi during growth is often important for development. In 1937, Robbins, as a result of a comprehensive study, was able to classify fungi into four groups according to their nitrogen requirements as follows: (*a*) those fungi able to fix atmospheric nitrogen and also able to use organic and inorganic sources of the elements, (*b*) those which use only organic nitrogen and also ammonium and nitrate nitrogen, (*c*) those which use only inorganic and ammonium nitrogen, and (*d*) those which use only organic nitrogen. Although such classification is to a large extent arbitrary, it nevertheless becomes of the greatest importance to study the nitrogen source in any analysis of the growth requirements of a given fungus.

The first factorial experiment indicated conclusively that K_2HPO_4 and $MgSO_4.7H_2O$ were necessary for good growth of *M. hiemalis*, and in order to obtain a high yield in terms of dry weight under the conditions of the experiment these salts should be given at concentrations of 0·012 M and 0·0015 M respectively. Hence in the second factorial experiment described below the salts were used at these concentrations for both (+) and (−) strains, although no statistical significance was found for the (−) strain as regards the utilization of $MgSO_4.7H_2O$. In this second experiment the object was to evaluate the amount of growth and to see whether a balance of salts is important when the nitrogen source is NH_4NO_3. Preliminary work showed this salt to be favourable for the growth of the fungus used; it is important to note, however, that both component radicles can supply nitrogen to the fungus.

Table 3. *Growth responses of* Mucor hiemalis (+) *and* (−) *strains to varying concentrations of* K_2HPO_4, NH_4NO_3 *and* $MgSO_4$

Results given as mg. dry weight

NH_4NO_3	Mean weight +	Mean weight −	$MgSO_4$	Mean weight +	Mean weight −
0·0125 M	44·49	52·20	0·00075 M	50·5	50·42
0·025 M	46·92	47·42	0·0015 M	49·91	53·49
0·0375 M	56·11	54·35	0·0030 M	47·12	50·07

	NH_4NO_3					
	0·0125 M Mean weight		0·025 M Mean weight		0·0375 M Mean weight	
$MgSO_4$	+	−	+	−	+	−
0·00075 M	45·98	54·92	47·93	46·42	55·82	49·91
0·0015 M	40·66	50·58	45·38	47·48	65·46	62·40
0·0030 M	46·83	51·09	47·47	48·35	47·06	50·76

	K_2HPO_4 0·008 M Mean weight		K_2HPO_4 0·012 M Mean weight	
	+	−	+	−
NH_4NO_3	45·76	48·74	52·40	53·91
0·0125 M	42·02	46·30	46·96	58·09
0·025 M	41·72	47·34	52·13	47·09
0·0375 M	53·45	52·17	58·11	56·54
$MgSO_4$				
0·00075 M	43·98	45·51	55·17	52·33
0·0015 M	48·25	52·25	52·75	54·73
0·0030 M	44·96	45·46	49·28	54·67

	NH_4NO_3											
	0·0125 M Mean weight				0·025 M Mean weight				0·0375 M Mean weight			
K_2HPO_4	0·008 M		0·012 M		0·008 M		0·012 M		0·008 M		0·012 M	
	+	−	+	−	+	−	+	−	+	−	+	−
$MgSO_4$												
0·00075 M	39·42	50·22	52·54	59·64	40·76	45·94	55·14	46·9	51·76	49·36	57·88	50·46
0·0015 M	39·23	41·98	45·78	59·18	42·76	53·98	48·0	40·98	66·84	60·38	54·58	64·02
0·0030 M	51·10	45·70	42·56	55·48	41·64	43·30	53·88	53·4	42·14	46·38	51·98	55·14

The experimental procedure in this second analysis was similar to that in the first. K_2HPO_4 was used at two levels, 0·008 M and 0·012 M; $MgSO_4.7H_2O$ was used at three levels, 0·00075 M, 0·0015 M and 0·003 M; while NH_4NO_3 was used at three levels, 0·0125 M, 0·025 M, and 0·0375 M. The results of the experiment expressed as a summary of the mean dry weights are given in Table 3, and the analysis of variance is seen in Table 4. The probability levels are again 99 : 1 and 19 : 1. Detailed consideration of the results is given below.

Dipotassium hydrogen phosphate; the analysis of variance shows that the results for both strains of the fungus are significant. The 0·012 M solution again gave the best dry weight, but the mean dry weight for the 0·008 M solution was greater than in Experiment 1.

Magnesium sulphate; the response of the two strains differed greatly. For the (+) strain, while the actual dry weights are little different from one another at the levels of the salt, the analysis of variance indicates significance. The highest of the dry weights is given by the lowest level of the salt, i.e. by 0·00075 M. The results for the (−) strain are not significant.

Ammonium nitrate; increasing concentrations of this salt produced significantly increasing growth responses for the (+) strain. Again the (−) strain behaves rather differently but the *F* value is still significant.

Interaction of salts of the second analysis

The results for the physiological interaction of K_2HPO_4 and $MgSO_4 . 7H_2O$ are highly significant. The greatest mean dry weight was obtained with 0·012 M-K_2HPO_4 when the concentration of $MgSO_47 . H_2O$ was at its lowest level. This result agrees closely with that of the first factorial experiment and indicates that it is a balance between these two salts which is important and not the concentration of either one of them. Thus a decrease in $MgSO_4 . 7H_2O$ without a corresponding increase in K_2HPO_4 would not have resulted in an increase of dry weight. The results for the (−) strain are not statistically significant, but the average yields for the various concentrations are of the same order as those for the (+) strain.

Table 4. *Analysis of variance. Exp.* 2

			F		
				Required odds	
(+) strain	D.F.	Mean square	Found	99 : 1	19 : 1
Total	89	97·09	—	—	—
K_2HPO_4	1	1002·0	39·92	7·01	3·98
$MgSO_4$	2	91·55	3·65	4·92	3·13
NH_4NO_3	2	1058·9	42·19	4·92	3·13
$K_2HPO_4 \times MgSO_4$	2	744·8	29·67	4·92	3·13
$K_2HPO_4 \times NH_4NO_3$	2	78·82	3·14	4·92	3·13
$NH_4NO_3 \times MgSO_4$	4	446·03	17·77	3·6	2·5
Error	76	25·1	—	—	—
(−) strain					
Total	89	120·09	—	—	—
K_2HPO_4	1	601·14	6·1	7·01	3·98
$MgSO_4$	2	111·52	1·2	4·92	3·13
NH_4NO_3	2	383·69	3·9	4·92	3·13
$K_2HPO_4 \times MgSO_4$	2	95·54	0·9	4·92	3·13
$K_2HPO_4 \times NH_4NO_3$	2	293·85	3·0	4·92	3·13
$NH_4NO_3 \times MgSO_4$	4	220·56	2·3	3·60	2·5
Error	76	97·84	—	—	—

The analysis of variance for the interaction of K_2HPO_4 and NH_4NO_3 shows significance at the lowest level of probability. The interaction is such that the best dry weight due to increased phosphate was only obtained with the highest concentration of NH_4NO_3. The results for the (−) strain again are not significant, but again the average yields are high and of the same order as those for the complementary strain.

In the results of the first-order interaction of NH_4NO_3 and $MgSO_4.7H_2O$ a high *F* value was obtained and all the dry weights were well above the average dry weights for other interactions and salts used. Table 3 shows that a very high dry weight of 65·46 mg. is obtained with 0·0375 M-NH_4NO_3 and 0·0015 M-$MgSO_4$. It is also seen that in the second order interaction the concentration of K_2HPO_4 does not greatly influence the relationship between NH_4NO_3 and $MgSO_4.7H_2O$. The results for the (−) strain are also significant. The combination of 0·0375 M-NH_4NO_3 and 0·0015 M-$MgSO_4$ gave the highest dry weight, and the concentration of K_2HPO_4 again does not seem to affect the response in respect to the balance between these two salts.

The analysis of the two factorial experiments described so far enables us to conclude (*a*) that a balance between the salts used is desirable, and (*b*) that the following solution will yield a high dry weight when supplied to either the (+) or the (−) strain of *M. hiemalis*: 0·0375 M-NH_4NO_3, 0·012 M-K_2HPO_4 and 0·0015 M-$MgSO_4.7H_2O$. The balance between the salts falls into two categories, firstly that between K_2HPO_4 and $MgSO_4.7H_2O$ and secondly that between $MgSO_4.7H_2O$ and NH_4NO_3. Initially a high level of phosphate is necessary to increase the dry weight, but given that high level the highest growth response is obtained when the balance between the other two salts is correct.

Third factorial analysis

In the experiments already described carbon was supplied as glucose at the 2% level. It was possible that an increase in the concentration of the carbon source might improve the yield estimated as dry weight. Hence an experiment was designed in which two levels of NH_4NO_3 were combined with three levels of glucose. Thus NH_4NO_3 was supplied in concentrations of 0·025 M and 0·0375 M and glucose at 2, 4 and 6% levels. The summary of the results of the experiment is given in Table 5.

From these results it is obvious that the limiting factor at all levels of glucose concentration is the NH_4NO_3 concentration. But at the higher sugar levels,

Table 5. *Summary of the response of* Mucor hiemalis (+) and (−) *strains to concentrations of glucose and ammonium nitrate*

Results given as mg. dry weight

		Glucose %		
Strain	Ammonium nitrate	2	4	6
(+)	0·025 M	48·0	26·6	30·2
	0·0375 M	64·5	62·1	64·5
(−)	0·025 M	40·9	27·1	29·4
	0·0375 M	64·0	55·9	59·8

e.g. 4 and 6 % the ill-effects of decreasing the NH_4NO_3 concentration was much greater than at the lower glucose level. Thus the dry weight of the (+) strain with 0·025 M-NH_4NO_3 and 6 % glucose is 30·2 mg., whereas with 2 % glucose it is 48·0 mg. compared with the dry weights with 0·0375 M-NH_4NO_3 and 2 % or 6 % glucose which were both 64·5 mg. There are two possible explanations of this result. Either the rate of utilization of NH_4NO_3 is increased with the high glucose concentration and so a low level of NH_4NO_3 becomes a limiting factor, or the increased glucose leads to an increased rate of respiration. In view of the fact that such a low dry weight is obtained the latter explanation is perhaps more likely. Table 5 indicates that 2 % glucose combined with 0·0375 M-NH_4NO_3 gave a satisfactory dry weight for (+) and (−) strains alike.

Fourth factorial analysis

Talley & Blank (1941) showed that the important factor governing nutrition in their experiments was the ratio between nutrients and not their absolute quantity. Thus an increase in any salt, if accompanied by a corresponding increase in the other constituents of the media, did not result in a change of dry weight provided the necessary balance was maintained. This fourth factorial experiment was designed to see whether or not this held for *M. hiemalis*. The absolute quantities of the salts found to be suitable for growth were doubled and halved, the ratio of one salt to another being maintained. The results expressed as average dry weights are given in Table 6.

Table 6. *Dry weights of* Mucor hiemalis (+) *and* (−) *strains when quantities of salts were doubled and halved*

Results given as mg. dry weight

NH_4NO_3	$MgSO_4$	K_2HPO_4	(+) strain	(−) strain
0·01875 M	0·00075 M	0·006 M	76·12	50·4
0·0375 M	0·0015 M	0·012 M	64·48	64·02
0·075 M	0·003 M	0·024 M	108·02*	81·86*

* Insoluble phosphate was deposited in the hyphal mesh and is included in these weights.

Again a difference in response between the (+) and (−) strains is seen but the final dry weights obtained when both strains were grown in the medium which had been shown to be well balanced for both the first order interactions were practically the same; there is an average difference of only 0·46 mg. The remainder of the results shows an inconsistency which was not unexpected. When the absolute quantities of the salts were balanced there was a marked increase in growth of the (+) strain but there was a characteristic decrease in growth of the (−) strain. When the absolute quantities of the salts were doubled, it was found that insoluble crystals of a phosphate formed within the hyphal mesh which of course vitiated the results at this high concentration. But it is probably true that there was no actual decrease in growth and possibly a little increase above the 64·5 mg. obtained for the original combination of salts.

DISCUSSION AND CONCLUSION

Experiments of this kind enable one to draw several conclusions which give positive information about the nutritional requirements of the fungus concerned. Although there is no dearth in the literature of examples showing the importance of individual salts and other chemical compounds to fungal growth, there are too few comprehensive analytical reports on this aspect of nutrition. Thus it is probably too early to make generalizations which the future may show to be invalid. It may be concluded that an analysis of the experiments described in this paper shows that at 20° and with trace elements supplied as impurities in the chemicals used a liquid medium containing 2% glucose, $0\cdot0375\,\text{M-}NH_4NO_3$, $0\cdot0015\,\text{M-}MgSO_4.7H_2O$ and $0\cdot012\,\text{M-}K_2HPO_4$ represents a fairly balanced medium and provides the best combination for a good strong growth of *M. hiemalis*. However, while a balance between the salts is necessary for good growth it is impossible to conclude that balance alone is the factor of over-riding importance in the nutrition of this species of Mucor, since a change in quantity whilst keeping the balance the same, as in Exp. 4, gave rather inconsistent results which could not be related to any failure of experimental technique. Thus a halving of quantity gave increased dry weight for the (+) strain and an equally reduced dry weight for the (−) strain as compared with results at the levels used throughout the experiments. This conclusion does not agree with that of Talley & Blank (1941), who found that balance alone controlled the growth of the parasitic fungus *Phymatotrichum omnivorum*. It is perhaps significant to note that Mucor is a saprophytic fungus, and this difference in the importance of a balance of mineral nutrients between these two fungi may also be a measure of the difference in their modes of nutrition. The importance of NH_4NO_3 in the culture solution is obvious. It seems to serve as the key nutrient in the balance of salts, for the first-order interaction of this salt with each of the others resulted in a definite and precise increase of growth.

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(*Received* 6 *May* 1953)

Edward, D. G. ff. (1954). *J. gen. Microbiol.* **10**, 27–64

The Pleuropneumonia Group of Organisms: a Review, together with some New Observations

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INTRODUCTION

The organisms of the pleuropneumonia group are of particular interest because, although they have distinctive properties, they resemble in certain respects other widely different organisms. They can exist as particles *c.* 150 mμ. in diameter, i.e. about the same size as the elementary bodies of vaccinia virus, but are nevertheless capable of multiplication in cell-free media. Their relationship to the bacteria has recently been complicated by the finding that under unfavourable conditions many bacteria undergo variation to a so-called L-phase which may resemble morphologically and culturally an organism of the pleuropneumonia group. Cholesterol, which is an essential growth factor for organisms of the pleuropneumonia group, is not known to be required as a nutrient by any bacteria, but is necessary for the growth of certain protozoa and other micro-organisms.

Some organisms of the pleuroneumonia group cause disease and are of practical interest to the veterinarian. Contagious bovine pleuropneumonia is one of the most serious infections of cattle, and even now in some parts of the world causes losses which are difficult to prevent. Other strains have been incriminated as a cause of disease in man, but for this the evidence is much less definite.

Information about the group as a whole and about the individual species is still very incomplete. Much of the investigation has been too limited in scope. Considerable attention has been paid to the morphology of the organisms, but very much less consideration has been given to their physiology. Since the organisms were found to be filterable through candles which held back bacteria they were originally classed with the viruses; many of the strains isolated later were encountered accidentally during the passage of viruses in laboratory animals, and so it happened that they were at first largely studied by the methods used for viruses. More complete examinations were neglected also because of technical difficulties. However, these difficulties can be overcome and the organisms have shown themselves amenable to study by most of the usual bacteriological techniques. Already physiological studies have yielded interesting results and in this review an attempt will be made to reconsider the pleuropneumonia group of organisms in the light of these findings, in order to encourage more intensive studies. These may reveal information of value in the understanding of bacteria and viruses, since the pleuropneumonia group of organisms share properties with both of these, and may also help in establishing the taxonomic relationships of the pleuropneumonia group.

HISTORICAL OUTLINE

It was recognized by Pasteur that contagious pleuropneumonia of cattle was caused by a specific infective agent which could not be seen under the microscope and did not grow in ordinary nutrient broth. In 1898 Nocard & Roux succeeded in growing the agent in a cell-free medium. Its peculiar pleomorphic morphology was described in 1910 by Bordet and also by Borrel and his colleagues, who named it *Asterococcus mycoides*. It was filterable through a Berkefeld V filter, and Elford (1929), using Gradocol filters, showed that cultures contained particles 125–150 mμ. in size. The organism was therefore usually regarded as a virus, and for nearly 25 years occupied a unique position amongst micro-organisms, because, although it had the dimensions of a virus, it was capable of multiplication in cell-free media.

In 1923 Bridré & Donatien showed that contagious agalactia of sheep was caused by an organism similar in morphology and cultural behaviour; this organism was later named *Anulomyces agalaxiae* (Wroblewski, 1931). The morphology and biochemical properties of these two 'viruses' were investigated by a number of workers (Holmes & Pirie, 1932; Ledingham, 1933; Klieneberger, 1934), but it was not until 1934 that another similar organism was isolated by Shoetensack from dogs suffering from distemper. In the following years similar organisms were isolated from a variety of sources; because they resembled the organism of bovine pleuropneumonia, they were called 'pleuropneumonia-like organisms' (PPLO).

Klieneberger (1935) isolated from cultures of *Streptobacillus moniliformis* a 'pleuropneumonia-like organism' which she called L1. At first she regarded it as a symbiont associated with the bacillus, but later (Klieneberger-Nobel, 1949) she agreed with Dienes (1947*a*) that it was a variant of the bacillus. Klieneberger and her associates (Klieneberger & Steabben, 1937; Findlay *et al.*, 1938, 1939*a*, *b*) also isolated pleuropneumonia-like organisms directly from rats and mice. Strains with different pathogenic and antigenic properties were isolated from mice by Sabin & Johnson (1940*b*). Meanwhile, Laidlaw & Elford (1936) had isolated from sewage pleuropneumonia-like organisms which differed from those isolated from animals by their ability to grow on simpler media without enrichment with serum, and by their lack of pathogenicity for animals. Seiffert (1937*a*, *b*) found similar saprophytic strains in soil and decomposing vegetable matter.

A claim by Swift & Brown (1939) to have isolated pleuropneumonia-like organisms from human cases of rheumatic fever aroused interest, but it was later realized that the organisms had been picked up from mice into which the rheumatic exudates had been injected. Subsequent attempts to isolate the organism directly from cases of rheumatism were negative (Sabin & Johnson, 1940*a*). Organisms of the group were, however, shown to inhabit the human genital tract (Dienes, 1940).

Progress was therefore rapid; whereas in 1934 only two members of the group were known, by 1941 it was realized that there existed a group of organisms, widely dispersed in nature and consisting of numerous species, some pathogenic, some saprophytic, with unique properties which distinguished them from bacteria, rickettsiae and viruses. The available information was excellently summarized by Sabin (1941*a*) in a review which should be consulted for details of the earlier work.

GENERAL CHARACTERISTICS

Definition

The criteria used by Sabin (1941*a*) to decide the admission of an organism into the pleuropneumonia group were: '(1) growth in cell-free culture media with the development of polymorphic structures including, "rings", globules, filaments, and minute filterable elementary bodies, usually 125–250 mμ. in size, which are the minimal reproductive units; (2) the development on suitable solid media of characteristic minute colonies which may be as small as 10–20μ. and as a rule not larger than 600μ.'. Dienes & Weinberger (1951) pointed out that this definition could be interpreted to include the L-phase variants of bacteria. Edward (1950*b*, 1953*a*) suggested that the L-phase organisms ought to be excluded from the pleuropneumonia group, because there appeared to be differences between them and organisms of the pleuropneumonia group proper in colonial appearances, in growth requirements and possibly even in morphology. The relationship of the L-phase organisms to the pleuropneumonia-like organisms will be discussed later (pp. 32–33), and attention will be drawn to those properties which appear to distinguish an L-phase organism from an organism of the pleuropneumonia group.

The reviewer agrees with Sabin that, although organisms of the pleuropneumonia group share many common properties, admission to the group should depend only on the characteristic morphology and colonial appearance. It would be unwise to be more specific than Sabin in describing the morphology, for interpretation of the microscopical appearances is still controversial. It is, however, possible to be more definite about colonial appearances which differ little from species to species. After 3 days of incubation colonies are translucent and circular with an entire edge, the surface is smooth and raised, sometimes with a small central elevation or depression. Well-isolated colonies of some species reach a diameter of 1·5 mm., but they usually measure 150–300 μ. and are often smaller. Owing to their small size they are best examined with a dissecting microscope (×10 magnification) using transmitted light. The surface is seen to be faintly marked and there is a central spot of variable size sometimes brown in colour, caused by down-growth into the medium. The colonies of a few species, including the L3 organism from rats (see p. 50) and the α and β strains from dogs (see pp. 49–50), have slightly different appearances to be described later. Exclusion of L-phase organisms from the pleuropneumonia group has been attempted by this description of colonial appearances, but in the opinion of the reviewer the definition of the pleuropneumonia group should include the statement that any organism known to be the L-phase of a bacterium, must not be included in the group.

Classification and nomenclature

The first organisms of the pleuropneumonia group to be isolated were given names; the organism of bovine pleuropneumonia in fact received at least four different names, each based on a different morphological interpretation of the nature of the organism. Symbols were later used to designate the numerous strains, mostly isolated from rodents and identified by their pathogenic and antigenic properties. Sabin (1941*a*) alone attempted a comprehensive classification in which scientific names were given to all the recognized species. Criticisms were immediately raised against his nomenclature; some of the objections had obvious validity, and Sabin (1941*b*) himself soon suggested a revised nomenclature, but this has not been generally accepted.

Sabin considered that organisms of the pleuropneumonia group differed fundamentally from bacteria, particularly in their method of reproduction. He therefore assigned them to a new class Paramycetes, alongside the Schizomycetes. The whole group was included in one order Anulomycetales divided into two families, one containing species isolated from animals and the other containing the saprophytic strains. The different species were grouped together into genera according to their animal host. Previously, Ledingham (1933) had suggested putting the two species known at that time into a new genus to be set up in the existing family of Actinomycetaceae. A third suggestion was to assign them to a new order Borrelomycetales (Turner, 1935*a*).

Thus the proposals made for classifying the organisms varied from placing them in a new genus of an existing family of bacteria to separating them altogether from bacteria into a new class. Because Dienes & Weinberger (1951) believed that the various strains had been derived in the course of evolution from different bacteria, they suggested that the organisms belonged to a morphological group of miscellaneous origin, somewhat analogous to the Fungi Imperfecti, and should not be assigned to one order or family in a natural system. Agreement on classification and nomenclature can be reached only after deciding their relationship to bacteria, including the L-phase variants of bacteria. When sufficient evidence is available for a decision to be made the problem should be considered by the International Committee on Bacteriological Nomenclature, so that an agreed system of nomenclature may be set up internationally, thus avoiding the repeated changing of names which has caused confusion in other fields. Meanwhile, it is desirable that newly described species should be given symbols rather than proper names. It will thus be possible to avoid the laws of priority fixing names which may later appear unsuitable. Sabin regarded his nomenclature as provisional and did not wish the names he suggested to be fixed by the laws of priority.

Agreement on nomenclature should not be delayed longer than is necessary because there is an urgent need for a less cumbersome term than 'organism of the pleuropneumonia group'. 'Pleuropneumonia-like organism' is often used, but cannot be translated directly into other languages, its abbreviation 'PPLO' also being only applicable in English. Moreover, the use of this term draws an unnecessary distinction between the organism of bovine pleuropneumonia itself and the other species of the group. The term 'L organism' is sometimes used because Klieneberger named some of her original strains L1, L2, L3 and so on. Although this has the advantage of brevity, it should be avoided as it refers only to a provisional system of labelling of strains by one worker. Its use, moreover, has led to considerable confusion because the L1 organism is now recognized to be a variant of a bacillus (*Streptobacillus moniliformis*), whereas L3, L4 and L5 of Klieneberger were members of the pleuropneumonia group proper, isolated directly from animals. Klieneberger-Nobel (1947) later called pleuropneumonia-like variants of other bacteria 'L1 cultures'. Other workers omitted the 'one' and spoke of 'L-type cultures' and 'L forms' (Dienes, 1948; Tulasne, 1951). The prefix 'L' has thus come to be associated with these variants, and in the opinion of the reviewer it should be restricted to them. There will then be no suggestion that, for instance, the L3 organism or the organism of bovine pleuropneumonia are variants of bacteria. Confusion has also arisen because 'L-form' and 'L-body' have been used to describe morphological changes in bacterial cultures, even when attempts to subculture a pleuropneumonia-like L-phase variant have failed. The changes to 'L-forms' or 'L-bodies' may represent L-phase variation, but on the other hand may be degenerative.

There are two subdivisions of the pleuropneumonia group, one containing strains believed to be saprophytic and capable of growing at 22° and on simple media, and the other containing strains which will not grow at 22° and which require media enriched with serum or ascitic fluid. There are a number of species with different pathogenic and biochemical properties, each distinct antigenically. The organism of bovine pleuropneumonia is the type species; it was called *Asterococcus mycoides* by Borrel and his colleagues (1910), and the rules of priority appear to make the name valid. The choice by Sabin (1941*b*) of *Anulomyces agalaxiae* (the organism of contagious agalactia), as the type species from which to build a system of nomenclature, was perhaps unfortunate in that its properties have been much less studied. Moreover, the organism of bovine pleuropneumonia, the first to be isolated, has already given its name to the group.

Relationship to the L-phase variants of bacteria

When cultures of many different species of bacteria have been exposed to unfavourable conditions, atypical colonies have appeared, composed of organisms which had lost their usual bacillary form. Subculture from such colonies was sometimes successful and could be maintained in series. These atypical organisms are now believed to be variants of the bacteria or a stage in their life cycle. In morphology and colonial appearance these variants or 'L-phase' organisms closely resemble organisms of the pleuropneumonia group. The L1 organism, the first L-phase variant isolated, was originally regarded by Klieneberger (1935) as a pleuropneumonia-like organism which occurred as a symbiont in cultures of *Strep. moniliformis*. Tulasne (1951) believed that an L-phase organism was indistinguishable from an organism of the pleuropneumonia group. Dienes & Weinberger (1951), in a recent review, emphasized that colonies of certain L-phase organisms may appear identical with those of true pleuropneumonia-like organisms.

The similarities, however, between bacterial L-phase organisms and organisms of the pleuropneumonia group may have been overemphasized. Ørskov (1942) described morphological differences between them; these differences were confirmed by Freundt (1950), who did not consider that the L-phase organisms belonged to the pleuropneumonia group. Three L-phase organisms, studied by the present author, differed in their colonial appearance from organisms of the pleuropneumonia group (Edward, 1950*b*; 1953*a*). The colonies of the L-phase organisms were more opaque and their surfaces were more heavily marked than the colonies of the pleuropneumonia-like organisms and they appeared to combine both pleuropneumonia-like and bacterial characteristics; the bacterial characters were even more marked in cultures in fluid and semi-solid media. The published descriptions suggest that cultures of other L-phase organisms also differed from those of true pleuropneumonia-like organisms. Dienes & Weinberger (1951), although they claimed that a colony of a salmonella L-phase organism might be indistinguishable from that of an organism of the pleuropneumonia group, stated that colonies grew to a diameter of 2 mm. and that sometimes growth 'progresses for several weeks and large opalescent colonies develop comparable in abundance to bacterial colonies'. Such colonies could be distinguished from those of true pleuropneumonia-like colonies.

In the opinion of the reviewer a culture can be identified as an L-phase organism when it is examined both with the naked eye and with low magnification at all stages of growth; further assistance is provided by cultures in fluid and semi-solid media. When repeatedly subcultured in penicillin-free media most L-phase organisms tend to revert to the bacillary phase, especially in fluid and semi-solid media. The first few subcultures of an L-phase organism are difficult and often unsuccessful, whereas those of an organism of the pleuropneumonia group are usually easy on a suitable medium. Klieneberger-Nobel* (1954) also draws attention to these differences between L-phase variants of bacteria and organisms of the pleuropneumonia group proper, and claims that they can be distinguished from each other.

The demonstration that bacteria can exist in an L-phase led to speculation about the nature of those pleuropneumonia-like organisms which exist independently of bacteria. Tulasne (1951) believed that organisms of the pleuropneumonia group were 'des formes L des bactéries fixées et adaptées depuis plus ou moins longtemps à un organisme vivant'. Although Dienes & Weinberber (1951) admitted the existence of an independent group of pleuropneumonia-like organisms, they suggested that the organisms had evolved in the past from bacteria by variation to an L-phase which had become stable and persistent, this change representing a simplification of structure.

An apparent similarity in growth requirements seemed at one time to support the notion that organisms of the pleuropneumonia group and L-phase organisms were closely related. All except the saprophytic strains of the pleuropneumonia group need media enriched with serum or ascitic fluid and so do the L-phase variants of Gram-negative bacteria. Subsequent investigation, however, has indicated an important difference in growth requirements between the two groups of organisms. Cholesterol, or certain other sterols, appears to be necessary for the growth of organisms of the pleuropneumonia group, but not for the growth of L-phase organisms (Edward & Fitzgerald, 1951*b*; Edward, 1953*a*). Two L-phase organisms grew on a medium in which serum had been replaced by a serum albumin fraction + an acetone-insoluble lipid fraction of egg yolk, whereas the further addition of a cholesterol suspension to the medium was required to permit the growth of typical organisms of the pleuropneumonia group. If these findings are confirmed by observations on other L-phase organisms, they suggest a fundamental difference between the two groups of organisms, and it would appear wise to be cautious in speculations upon the origin of the pleuropneumonia group.

Some organisms of the pleuropneumonia group ferment carbohydrates; others appear to lack this power. All the strains with fermentative capabilities tend to form acid from the same carbohydrates; all ferment glucose, maltose, dextrin, starch and glycogen, and some strains also ferment fructose, galactose and mannose (Table 1). No strain was found capable of fermenting the other carbohydrates tested, including lactose, saccharose, mannitol and dulcitol (Edward, 1950*b*). This similarity in fermentative power suggests that the various organisms of the group are closely related to each other. If each species had been derived from a different bacterium and was a stable and persistent form of L-phase variant, as Dienes & Weinberger suggested, the species might be expected to differ from each other in fermentative capabilities, because a bacterium in the L-phase has been observed to ferment the same carbohydrates as it did in the bacillary phase.†

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* I am indebted to Dr Klieneberger-Nobel for letting me see her review before it was published.

† See Addendum (p. 64).

Technical methods

Organisms of the pleuropneumonia group are delicate, their nutritional requirements are exacting and their growth is comparatively slow and sparse. These factors make investigations technically difficult, but the difficulties can largely be overcome and the organisms examined by most of the usual bacteriological and serological methods, with certain modifications in technique, provided that adequate amounts of media are available. In order to encourage the complete examination of strains which may be isolated in future a brief description of suitable techniques will be given.

A satisfactory culture medium is clearly essential; its composition will be discussed later. For primary isolation, material (e.g. swabs from the vagina) can be sown directly on solid media; there appears to be no advantage in preliminary cultivation in a fluid medium. Cultivation in a semi-solid medium (Beveridge, 1943) is useful, especially when the organisms in the inoculum are few or are poorly adapted to the medium, but fluid media are not to be recommended except to obtain suspensions. The plates must be incubated in a moist atmosphere, which can be obtained by placing them in a closed vessel containing an exposed piece of moist cotton-wool. Most strains grow satisfactorily under aerobic conditions, but some require anaerobic (or microaerophilic) conditions or an atmosphere of air + 10 % CO_2 (Dienes, Ropes, Smith, Madoff & Bauer, 1948; Ruiter & Wentholt, 1952).

In the past, contamination of cultures during prolonged incubation in a moist atmosphere was a frequent difficulty, but this has been overcome by incorporating bacteriostatic substances in the media. A selective medium containing thallium acetate and penicillin permitted the isolation of pleuropneumonia-like organisms in pure culture from highly contaminated material (Edward, 1947*b*). Smith, Morton & Leberman (1950), wishing to avoid penicillin, were successful with a mixture of crystal violet and potassium tellurite. White (1952) used a mixture of thallium acetate, sulphamezathine and crystal violet.

Colonies appear on solid medium in 2–4 days, but plates should be kept 6 days before being discarded as negative. The colonies, although usually visible to the naked eye, are best identified by their characteristic appearance by means of a dissecting microscope (× 10 magnification) and slightly oblique transmitted light. Examination of impression films stained by Giemsa's method supplies confirmation (Klieneberger-Nobel, 1954). In the opinion of the reviewer examination with a dissecting microscope provides not only the easiest but the most reliable method of diagnosis. It is not an advantage to obtain greater magnification with the two-thirds objective of a microscope. The dissecting microscope, which has a greater depth of focus, gives a more composite picture of a colony. When impression films only are relied on, degenerative or L-phase changes in bacterial colonies may lead to mistakes. Bacteria are particularly liable to form L-phase colonies when penicillin is present in the medium (Dienes, 1947*b*). In preparations examined by dark-ground microscopy pleuropneumonia-like organisms are mimicked by a number of objects, including red cell envelopes and lipid globules.

Subculture should be made from a single colony by means of a Pasteur pipette drawn to a fine capillary point. Subsequent subcultures may be successful with an ordinary loop, but it is preferable to cut out a piece of agar culture, invert it and streak it over the medium to be inoculated. It should be remembered that some species, including the human genital strains, are easier to handle than others and that techniques which succeed with the easier strains may prove unreliable with the more difficult ones. Many strains are delicate and die when cultures are kept for more than a day or two on the bench or at 4°. The only safe way of maintaining them on solid media is by subculture twice weekly. As this is laborious they are best preserved for long periods by drying from the frozen state or by storage at −20°.

Fermentation of carbohydrates has been demonstrated by inoculating serum agar plates containing 1 % carbohydrate and 0·005 % phenol red. To detect haemolysis the organism was grown for 2 days on a serum agar plate; then a thin layer of the same medium containing 5 % of a horse red blood cell suspension was poured over the surface and incubation was continued for a further 2 days. Alternatively, the organism was seeded into serum broth and at daily intervals afterwards 1 ml. of culture was removed. To this sample two drops of a 5 % suspension of horse red cells was added and the mixture was incubated at 37° for 5 hr., with shaking at half-hourly intervals. Changes in colour of the erythrocytes were noted (Edward, 1950*b*).

Suspensions of organisms suitable for serological and other investigations may be obtained by centrifuging broth cultures in the angle head for 1 hr. at 4000 r.p.m. In a fluid culture the maximum number of *viable* organisms is present just as opalescence first appears, or even before. As the culture becomes more opalescent on longer incubation, the viable elements decrease in number although the mass of antigenic material may increase for a time. A considerable economy in medium has been effected by growing the organisms in a layer of fluid on the surface of a solid medium. Serum agar was filled into 4 oz. flat screw-capped bottles, laid on their broad sides to provide a large surface. They were inoculated by pipetting on to the surface a suspension of organisms obtained by washing-off a 2- to 3-day growth on an ordinary plate with *c.* 2 ml. of nutrient broth. The bottles were incubated on their sides and gently rocked once each day. The fluid was finally pipetted off, the organisms in it being concentrated further by centrifugation when required (Edward, unpublished observations).

Nutritional and growth requirements

The first necessity for cultivation *in vitro* is a rich basal medium; most workers have used an ox-heart infusion broth with the addition of 1 % peptone. Some commercially available peptones were more satisfactory than others (Morton, Smith & Leberman, 1951). The addition of a red blood-cell suspension has been recommended, the medium being subsequently boiled and clarified by sedimentation (Klieneberger, 1936; Dienes, 1939). Beveridge (1943) added liver extract. Edward (1947*b*) noticed that growth on media enriched with horse serum varied according to the batch of serum used. When growth was poor, it was improved by adding a filtrate of a staphylococcal culture or a freshly prepared yeast extract. The growth factor in yeast was not coenzymes I or II (adenine nicotinamide dinucleotides; DPN and TPN). Two old laboratory strains were found to have become sensitive and were inhibited by high concentrations of yeast extract (Edward, 1950*b*). Glucose has been used to improve growth (Sabin, 1941*a*).

The minimum concentration of agar should be employed, since the growth of some strains is impaired by too stiff a gel. The different gelling strengths of different batches of agar cause difficulties. Although some strains grow over a fairly wide range of pH values, others grow best in an alkaline medium, and it is usual to adjust the final pH of the medium to a value between 7·6 and 8·0 (Edward, 1950*b*; Morton, Smith, Williams & Eickenberg, 1951). A few strains, which grew well anaerobically on media at pH 7·0 to 8·0, failed to grow aerobically on a medium at pH 8·0, but grew well aerobically on a medium at pH 6·5 (Edward, unpublished observations).

The nutritional requirements of the pleuropneumonia-like organisms are of special interest as they are the smallest organisms capable of growth in cell-free media. The requirements of the saprophytic strains, isolated from sewage (Laidlaw & Elford, 1936), are simpler than those of the rest of the group. These sewage strains grew in Hartley's broth without the addition of serum; Fildes's peptic digest of red cells improved growth and was essential for the growth of one strain (Pirie, 1937). All other organisms in the group require media enriched with materials such as serum or ascitic fluid; 20 % (v/v) of serum or 30 % (v/v) ascitic fluid is usually added, although good growth of well-adapted strains can be obtained with as little as 2·5 % serum. The sera from different species of animal are not equally effective; some sera appear to be inhibitory in high concentrations (Smith & Morton, 1951*a*; Freundt, 1952*a*). Horse and human sera are suitable for most strains. Many species of pleuropneumonia-like organisms will not grow on media enriched with rabbit serum, but some strains isolated from human saliva grow better on rabbit serum than on horse serum agar (Edward, unpublished observations).

Identification of the growth factors in serum, to allow the replacement of the serum in the medium by simpler or known materials, would seem to be a necessary preliminary to attempts to find a suitable chemically defined medium. When the lipid was extracted from horse serum, neither the lipid fraction alone nor the lipid-free fraction supported growth, but growth occurred on a medium containing both fractions (Edward & Fitzgerald, 1951*b*; Edward, 1953*a*). Serum could be replaced by adding a suspension of cholesterol + bovine albumin fraction V + an acetone-insoluble lipid fraction (AIL) of egg yolk. A degree of growth, which varied in amount with different strains, was obtained with cholesterol alone, cholesterol + starch, cholesterol + bovine albumin and cholesterol + AIL. There was no growth on media containing starch, bovine albumin or AIL, separately or together, without the addition of cholesterol. Strains differed in the relative proportions of cholesterol and AIL required for optimal growth. Excessive concentrations of one or the other were sometimes inhibitory. Cholestanol and stigmasterol promoted growth as well as cholesterol, but the cholesteryl esters of acetic, stearic and oleic acids were not effective.

These findings, which suggest cholesterol or another sterol or related compound to be essential for growth, must be reconciled with the findings of Priestley & White (1952) that the serum factor was heat stable and could be separated from the coaguable proteins, and with the claims of Smith & Morton (1951*a*, 1952) to have identified the serum growth factor as a protein of low molecular weight. The examination of a serum fraction, prepared according to Smith & Morton's methods and available commercially, however, showed it to contain lipid, including cholesterol (Edward, 1953*a*). After extraction of the lipid, this serum fraction no longer promoted growth. It thus seems likely that the preparations believed by Smith & Morton to consist only of protein owed their activity to the fact that they also contained lipid which included cholesterol. Haemoglobin solutions, which have been used to replace serum, probably contained lipids derived from the red cell stroma (Gilmore & Sprince, 1949; Smith & Morton, 1951*a*).

The action of serum in promoting growth thus appears to be complex, both protein and lipid fractions being required. The differences in the relative concentrations of cholesterol, phospholipid and protein in the sera of different species of animal probably explain why the sera differ in their ability to promote growth. Rabbit serum usually contains relatively little cholesterol. Strains which failed to grow on a rabbit serum medium grew on a medium containing rabbit serum + cholesterol. Protein and starch may act by binding fatty acid which would otherwise combine with the cholesterol. Oleic acid was shown to inhibit growth (Edward & Fitzgerald, 1951*b*). Attempts to identify the factor in AIL which improved growth were unsuccessful. Cholesterol is required by certain protozoa, e.g. *Trichomonas columbae* (Cailleau, 1937) and by a myxothallophyte *Labyrinthula vitellina* var. *pacifica* (Vishniac & Watson, 1953), but is not known to be an essential nutrient for any species of bacteria. Its requirement by organisms of the pleuropneumonia group is therefore interesting.

Some organisms of the pleuropneumonia group are unable to grow, at least in primary culture, unless another growth factor besides cholesterol is supplied. There was no growth when samples of bull semen were inoculated on the ordinary serum agar medium which was satisfactory for growing other pleuropneumonia-like organisms; positive cultures, however, were obtained from the semen after adding hog gastric mucin to the medium (Edward & Fitzgerald, 1952). Investigations in progress (Edward) suggest that the factor in mucin is deoxyribonucleic acid (DNA). These organisms from the bovine genital tract appear to be defective in their ability to synthesize DNA; in the animal body they probably depended upon the synthetic mechanisms of the host. They thus show a certain similarity to viruses which depend so extensively on the synthetic mechanisms of the host. It is not yet known at what stage in the synthesis of DNA the defect lies. The deoxyribosides of thymine and guanine did not promote growth, so the organism may need to be supplied with a more complex molecule or at least one containing phosphorus and deoxyribose.

Growth of certain other strains of pleuropneumonia-like organisms, in addition to the bovine genital strains, has been found to be improved at first isolation by DNA.

Since strains differ in their optimal growth requirements it is essential that nutritional studies should be carried out with a number of representative strains. It is possible that other organisms of the group exist which have not so far been isolated, because they require other unknown growth factors not present in the media in use or because the proportions of lipid and protein in the sera used for enrichment have been unsuitable.

Growth in the embryonated egg and in tissue culture

Growth has been obtained on the chorio-allantoic membrane of the living chick embryo. Some strains grew poorly and there was better growth after the embryo had been killed by chilling (Sullivan & Dienes, 1939; Swift, 1941). No characteristic lesions were produced, but *Asterococcus mycoides* sometimes caused death of the embryo (Tang, Wei & Edgar, 1936). The organisms were also grown in tissue culture (Sullivan & Dienes, 1939). Recently a strain of *A. mycoides* was found to have become less virulent after serial passage in the embryonated egg, while retaining its ability to immunize (Sheriff & Piercy, 1952). Vaccines prepared in eggs from this strain are being successfully used in the prevention of contagious bovine pleuropneumonia. *Anulomyces agalaxiae* has also been grown on the chorio-allantoic membrane and suspensions of the infected embryonic tissues have been used to prepare vaccines (Lopez & Lopez, 1952).

Morphology

The pleuropneumonia-like organisms are Gram-negative, but are not easily seen in films stained by Gram's method. Numerous techniques which use stained or unstained preparations have therefore been devised for their study. The fact that different workers have used different techniques possibly explains why they have described and interpreted the morphology so differently.

Cultures contain small particles, larger round bodies of various sizes, and filaments. The particles are the minimal reproductive units and can grow into the other forms. One, two or three filaments appear to grow out from a particle; they lengthen and later segment or break up into numbers of small particles, similar to the original particle. This was the only method of reproduction observed by Freundt (1952*a*, *b*), who regarded a mycelium, exhibiting true branching, as the important morphological feature, the large round bodies being involutionary and of poor viability. Freundt's work is of special interest as he combined experiment with morphological observation; his observations on the effects of nutrition on morphology need extending. The breaking up of filaments into particles, described by Freundt (1952*a*) and illustrated in his micrographs, resembles in many respects the breaking up of filaments, which had been extruded from the cytoplasm of infected cells, to form the elementary bodies of influenza virus (Wyckoff, 1953). Pulvertaft (1953), who made cinematographic records of growth, noted that the small particles appeared suddenly, apparently from empty space, as far as microscopical resolution was concerned; they were not formed by division of other particles.

Dienes (1945) drew attention to short rod-shaped forms, often showing bipolar staining, which were formed by segmentation of the filaments, and emphasized their likeness to rod-shaped bacteria. Bacillary forms were observed in electron micrographs; some had a cell wall (Weiss, 1944; Smith, Hillier & Mudd, 1948). Most forms of the organism, however, had no rigid cell wall and were therefore very plastic. Klieneberger & Smiles (1942) believed that the filaments were largely artefacts due to distortion of plastic organisms. They regarded the large round bodies as the important morphological feature. Dienes agreed with these workers that in addition to reproduction by binary fission, these round bodies provided another method of reproduction. Large numbers of particles appeared to develop within the cyst-like round body and were liberated after rupture of the membrane. Segmentation of some round bodies and the extrusion of filaments from others were observed in electron micrographs (Smith *et al.* 1948). This method of development of particles within a large body shows certain similarities to the intracellular multiplication of psittacosis virus. It may be called multipolar germination. Morphological differences have been noted between different species (Sabin, 1941*a*), but, since observations have usually been limited to single strains of a particular species, it is doubtful whether the appearances are sufficiently constant to distinguish the species. Andrewes & Welch (1946) observed a peculiar form of motility, apparently independent of the action of flagella, with certain mouse strains.

Less is known about the appearance of the organisms in the tissues of infected animals. The organisms are too small and pleomorphic to be easily distinguished from other types of granule, but forms similar to those seen in cultures have been observed in exudates (Turner, 1935*b*; Tang, Wei & Edgar, 1936); the organisms were there extracellular. It is less well established whether they also occur intracellularly. Intracytoplasmic granules in the mesothelial cells of the peritoneum of infected mice were considered by Sabin (1941*a*) to be the infecting organisms, but in other infections no cell inclusions were seen (Tang *et al.* 1936). Bacillary and coccal bodies were seen inside pus cells in an abscess produced in a mouse by a strain of human origin (Ruiter & Wentholt, 1952). Intracytoplasmic 'cocco-bacilliform bodies' were found in purulent exudates of mice suffering from infectious catarrh. Pleuropneumonia-like organisms were isolated from the latter lesions, and even after five subcultures they reproduced the disease. Cocco-bacilliform bodies were found in the pus cells of lesions produced by the cultures and they were therefore believed to represent the infecting organisms (Edward, 1947*a*). Similar cocco-bacilliform bodies have been found associated with infections of the upper respiratory tract of fowls and rats, but their relationship to pleuropneumonia-like organisms has not been determined (Nelson, 1935, 1940). Cell inclusions in smears from the human genital tract were described by Harkness (1950), who believed them to be infecting pleuropneumonia-like organisms, but no proof of this was offered.

The recent findings of Klieneberger-Nobel (1954) are of interest. Stained preparations of cultures of material from mice infected with pleuropneumonia-like organisms were made after a few hours of incubation and were examined to find out in which cells pleuropneumonia-like organisms were beginning to multiply to form colonies. Pleuropneumonia-like organisms were demonstrated in leucocytes and other phagocytic cells, but not in fixed tissue cells. Klieneberger-Nobel also showed that stainable bodies which could be mistaken for pleuropneumonia-like organisms appeared in pus cells as a result of degeneration.

Biochemical properties

Some species have biochemical activities which assist their identification (Edward, 1950*b*). Fermentation of carbohydrates with production of acid was demonstrated on solid media by a change in colour of phenol red; the results were somewhat different from those obtained with fluid cultures (Laidlaw & Elford, 1936; Warren, 1942). Some species haemolyse or discolour horse red blood cells (Warren, 1942; Edward, 1950*b*).

Cultures of certain species produce a peculiar 'pearly' film, together with small spots in the medium (Edward, 1950*a*). The spots consist of calcium and magnesium soaps; the composition of the film is more complex, but cholesterol and phospholipid have been detected in it. The formation of a film and spots were best noted on horse serum agar incubated for 6 days. On media enriched with egg yolk spots were formed by some strains which did not form them with horse serum. A clearing of the egg-yolk medium was produced, due to liberation of fatty acids (Edward, unpublished observations). These observations emphasize the importance of lipids in the metabolism of the pleuropneumonia-like organisms. The film and spots are possibly formed by the activity of lipases which break down phospholipid. Pirie & Holmes (1933) showed that the lipid phosphorus was decreased in the medium during growth of the organism of agalactia. The composition of the medium, particularly the proportions of protein and lipid and the amount of fatty acid already present, also determine whether a film and spots are developed on a particular medium by a particular strain.

Holmes & Pirie (1932) studied the utilization of glucose by *Asterococcus mycoides* and showed that this organism and the organism of agalactia reduced methylene blue; lactic acid was the only hydrogen donor identified (Pirie & Holmes, 1933). They subsequently examined the metabolic activities of two of the sewage organisms of Laidlaw & Elford (Holmes, 1937; Pirie, 1937, 1938). Unfortunately there has been no subsequent attempt to extend these observations. Further study of the enzyme systems possessed by these organisms is required, using techniques such as those described by Clarke & Cowan (1952), as this may not only reveal information of fundamental interest, but may also lead to the development of more biochemical tests for identifying species. The organism causing contagious pleuropneumonia of goats is particularly suitable for such studies, as it grows more vigorously than other strains. The observation made by Longley (1951) that this organism liquefied coagulated serum suggests that investigation into the proteolytic activities of the pleuropneumonia-like organisms should be made. Holmes & Pirie (1932) detected only a small increase in ammonia in the medium during growth of *A. mycoides* and the non-protein nitrogen, amino nitrogen and ammonia were not appreciably affected by growth of *Anulomyces agalactia* (Pirie & Holmes, 1933).

Organisms of the pleuropneumonia group, in common with the bacterial L-phase variants, possess two other properties which differentiate them from bacteria in the bacillary phase and probably reflect fundamental differences in their metabolism. One is a more or less complete resistance to the bacteriostatic action of penicillin without inactivation of the penicillin, e.g. by a penicillinase. A clue to the mode of action of penicillin might be afforded by determining how pleuropneumonia-like organisms differ from bacteria in their enzyme systems. The other property, which also probably reflects a peculiarity of metabolism, is the growth of surface colonies into the agar medium.

Other properties

Filterability. All ultra-filtration analyses which have been carried out on these organisms have shown that most of the organisms are removed by filtration through a Gradocol membrane of A.P.D. 0·8 μ, although the small particles pass membranes of successively smaller A.P.D. The size of the minimal reproductive elements was thus estimated; those of *Asterococcus mycoides* and of the sewage organisms measured 125–175 mμ., those of *Anulomyces agalaxiae* 150–225 mμ. (Elford, 1938; Laidlaw & Elford, 1936), and those of an organism causing pneumonia in mice 165–247 mμ. (Edward, 1940). Ultra-filtration analyses of other strains were reported by Findlay *et al.* (1938, 1939*b*) and by Sabin (1941*a*).

Resistance to physical and chemical agents. The organisms are very susceptible to heat. Some strains are killed by heating at 45° for 15 min., but others withstand 15 min. but not 30 min. at 45° and are killed by 15 min. at 55° (Edward, 1940; Sabin, 1941*a*). They resist freezing and have remained viable for at least one year at −20°. Cultures may also be preserved by freeze-drying. *Asterococcus mycoides* was quickly killed by ether and was lysed by bile salts (Tang, Wei, McWhirter & Edgar, 1935). Other organisms of the group were markedly susceptible to the bactericidal action of soaps (Keller, Smith & Morton, 1952). The inhibitory and lethal actions of various other chemicals were studied by Edward (1947*b*) and by Smith *et al.* (1950).

Sensitivity to chemotherapeutic agents. The first successful attempt at chemotherapy was made by Bridré, Donatien & Hilbert (1928) who showed that stovarsol had a preventive and curative action in contagious agalactia of sheep. Later, infection of rats by the L4 organism attracted interest, because it was thought that in spite of differences in pathology the resulting polyarthritis might provide an experimental approach to the treatment of rheumatoid arthritis in man. More recently, in view of the possibility that pleuropneumonia-like organisms may cause human genital infection, strains isolated from the genital tract have been tested against the new antibiotics.

Infections of rodents were prevented and cured by organic compounds of gold (Findlay *et al.* 1940), although the organisms were not inhibited *in vitro*

(Sabin & Warren, 1940*a*, *b*). Pleuropneumonia-like organisms were not affected by sulphonamides or by penicillin (Beveridge, 1943). Arthritis in rats caused by the L4 organism, although unaffected by penicillin treatment (Powell & Rice, 1944) and by cortisone (Kuzell & Mankle, 1950) was cured by streptomycin (Powell, Jamieson & Rice, 1946), by aureomycin (Kuzell, Gardner & Fairley, 1949) and by terramycin (Kuzell & Mankle, 1950). Human genital strains were inhibited *in vitro* by streptomycin, aureomycin, chloramphenicol and terramycin (Hatch, 1949; Leberman, Smith & Morton, 1950, 1952; Melén, 1951) and could no longer be cultivated from patients who had been treated with these anitbiotics (Dienes *et al.* 1948; Brown, Wichelhausen, Robinson & Merchant, 1949). The action of the antibiotics was bacteriostatic rather than bactericidal (Robinson, Wichelhausen & Brown, 1952). Strains varied in the degree of sensitivity and drug-resistance developed against streptomycin (Paine, Murray, Perlmutter & Finland, 1950). Streptomycin, which inhibited the organism of bovine pleuropneumonia *in vitro* (Mornet, Balis & Bachirou, 1949), appeared to act beneficially in the treatment of the disease. Novarsenbenzol was also active in controlling the infection in bovine pleuropneumonia and encouraging preliminary results were obtained with *p*-aminobenzenesulphonyl-(amino-2-methyl-diazine) (Mornet, Orue & Marty, 1951).

Immunological reactions

The repeated inoculation of rabbits with cultures of pleuropneumonia-like organisms induces the formation of antibodies which are capable of neutralizing pathogenic strains and can be demonstrated by agglutinin, precipitin and complement-fixation reactions. An immune rabbit serum specifically inhibited the agglutination of fowl red cells by a strain of pleuropneumonia-like organism isolated from embryonated hen eggs (Herick & Eaton, 1945). Antisera are also capable of inhibiting growth of the homologous organisms. Some antisera, when added to a horse serum agar plate at a dilution of 1/1000, completely inhibited growth (Edward & Fitzgerald, 1954). The highest dilution at which a serum was inhibitory was not always the same as the highest dilution which caused agglutination; one or other dilution might be greater. Inhibition of growth was unaffected by heating the sera to 56° and so was independent of complement. An antiserum only inhibited the homologous strains and those strains which were shown by the agglutination reaction to be serologically related. In fluid cultures organisms were not killed immediately by the addition of antiserum, although growth was prevented; even 24 hr. later living organisms were detected by subculture.

The growth inhibitory action of antibody has a practical use because the antigenicity of a strain can be tested with only a small amount of suspension. It is also of interest as it represents another difference between organisms of the pleuropneumonia group and bacteria; the latter do not appear to be inhibited by homologous antisera in the absence of complement. An antiserum against the L-phase variant of *Proteus vulgaris* inhibited growth of the L-phase but not that of the bacillary phase. Inhibition of growth shows some similarity to neutralization of a virus by antibody; in both cases combination of antigen with antibody prevents multiplication although it does not cause immediate death.

Klieneberger (1938, 1940) studied the agglutination reactions of a number of strains of pleuropneumonia-like organisms; she showed that strains of the same species shared common antigens and that there were only occasional cross-reactions at low titre between strains of different species. Subsequent work has confirmed that each species is distinct antigenically. When many strains of a particular species of pleuropneumonia-like organisms were examined antigenic differences between them were noted (Edward, 1950*a*; Edward & Fitzgerald, 1951*a*). Frequently a strain was found which was not agglutinated by an antiserum against a second strain and yet both these strains were agglutinated by an antiserum against a third strain. Species-specific antigens were also demonstrated by the complement-fixation test in strains which failed to agglutinate with a particular antiserum. The organisms thus appear to possess a highly complex antigenic constitution and agglutinin-absorption tests have failed to differentiate between group and type-specific antigens. No evidence has been obtained that the antigenic structure is labile and dependent on the conditions of culture.

Animals after recovery from natural and experimental infections are immune to subsequent infection, but antibodies are not always demonstrable in their sera. Agglutinins, precipitins and complement-fixing antibodies appear sufficiently regularly in contagious bovine pleuropneumonia to provide methods for laboratory diagnosis, but neutralizing antibodies were not found in the sera of infected mice and rats (Sabin, 1941*a*). The complement-fixation test has been successful in the diagnosis of contagious agalactia of sheep and goats (Zavagli, 1951). Complement-fixing antibodies were detected in the vaginal mucus of cattle which were harbouring pleuropneumonia-like organisms in the genital tract (Szabó, 1951). Priestley (1952) showed that the serum of cattle which had recovered from contagious pleuropneumonia or had been immunized against the disease contained antibodies capable of killing *Asterococcus mycoides* in the presence of complement *in vitro*, the effect being similar to bacteriolysis of certain bacteria.

Vaccination against bovine pleuropneumonia is effective only when a living vaccine is used. Cultures of *A. mycoides* attenuated by a sufficient number of serial subcultures in artificial media or by cultivation in embryonated eggs are now used as vaccines. A strain which has been attenuated too much will not confer adequate immunity. Protection against agalactia has been claimed by using as a vaccine cultures of *Anulomyces agalactia*, inactivated with formalin and adsorbed on aluminium hydroxide (Lopez & Lopez, 1952). Cultures inactivated by heating also protected mice against polyarthritis (Sabin, 1941*a*).

Pathogenicity

Four species of the pleuropneumonia group are known to produce natural disease; the three which are of veterinary importance cause, respectively, contagious pleuropneumonia of cattle, contagious agalactia of sheep and goats, and contagious pleuropneumonia of goats, all three being diseases with a high mortality. The fourth, the L4 organism, was isolated from rats with polyarthritis. Cultures are virulent for the corresponding animal host. The subcutaneous inoculation of *Asterococcus mycoides* produces a spreading and usually fatal cellulitis. The typical lung lesions of pleuropneumonia are not produced when animals are infected by the usual inoculation routes. They have, however, occasionally followed intratracheal injection of virulent lymph and were produced with greater regularity by allowing animals to inhale fine sprays or aerosols of cultures (Campbell, 1938). On repeated subcultivation in artificial media strains of *A. mycoides* lost their virulence. Intravenous injection of the L4 organism produced polyarthritis in rats and the virulence of some strains was retained even after years in artificial culture (Parkes, Wrigley & O'Brien, 1951); other strains quickly lost virulence with repeated subculture. The virulence of a culture appeared to be directly proportional to the speed with which it decolorized methylene blue (Warren, 1942). A strain which was unable to localize in the joints still produced local abscesses in rats and mice (Edward, 1950*b*).

Another group of strains are commensals in rodents. Animals carry the organisms for long periods but remain unaffected unless they are subjected to some experimental procedure. Pleuropneumonia-like organisms have even been isolated directly from brains of healthy mice (Sabin, 1941*a*). Cultures of the pleuropneumonia-like organisms isolated from rodents produced lesions after intravenous injection; some strains retained their virulence during more than 100 subcultures in artificial media (Sabin, 1941*a*). Cultures of other strains were only virulent when mixed with adjuvants such as agar or virus-infected brain tissue (Findlay *et al.* 1938, 1939*b*).

The pathogenicity of several species, including those isolated from the human and bovine genital tracts, is still doubtful (Edward, 1952). Since some undoubted pathogens lose virulence rapidly in subculture while other strains need adjuvants to enable them to produce lesions, investigations of pathogenicity are difficult. The L4 organism is known to be a commensal in rats; some unknown factor must determine whether it initiates polyarthritis. The importance of a second factor in initiating infections by organisms of the pleuropneumonia group was emphasized by Klieneberger-Nobel (1954), who studied the growth of pleuropneumonia-like organisms in the peritoneal cavity of mice infected with ectromelia virus. Organisms may be harmless in their normal habitat and yet produce disease when they reach some other part of the body. The retention of virulence during artificial cultivation may depend on the constitution of the medium.

Infectious catarrh of the upper respiratory tract of mice may be another disease caused by organisms of the pleuropneumonia group. It was reproduced by early subcultures of organisms isolated from lesions, but it is possible that another infective agent which had survived in the cultures caused the disease and produced the typical intracellular 'cocco-bacilliform bodies' (Edward, 1947*a*). The pathogenicity of strains isolated from fowl coryza is likewise unknown. Finally, the pleuropneumonia group includes species which have been found only as commensals, unassociated with disease.

Organisms of the pleuropneumonia group are believed to possess a considerable degree of host specificity. Possibly this conclusion has been based on rather scanty evidence because a wide range of animal species has not been used for testing the pathogenicity of many of the organisms. The most complete investigations have been made with *A. mycoides*; in some experiments cultures infected sheep, goats and buffaloes but not a number of other animals. Some of the rodent strains produced lesions after inoculation in rats and mice. Recently there have been reports that lesions were produced in mice with strains isolated from human infections. Mice inoculated intranasally with these organisms developed pneumonia (Grünholz, 1950; Carlson, Spector & Douglas, 1951), but in assessing the significance of this finding it must be remembered that some stocks of mice harbour strains capable of causing pneumonia (Edward, 1940). Strains isolated from cases of gangrenous balanitis produced purulent lesions after injection, together with agar, into the foot pads of mice (Ruiter & Wentholt, 1952).

The different infections by members of the pleuropneumonia group cause different tissue reactions. Bovine pleuropneumonia chiefly affects the connective tissue, causing acute inflammation with a profuse fibrinous exudate. In agalactia the connective tissue is also involved, but the inflammatory reaction is less acute and is followed by proliferation of fibrous tissue; abscesses are sometimes formed. Some of the strains isolated from mice produced proliferative inflammatory reactions after invading and multiplying within mesenchymal cells (Sabin, 1941*a*). The type A strains which multiplied in this way formed a filterable exotoxin which also had a destructive effect on cells of the brain. An exotoxin pathogenic for cotton rats was formed by the pleuropneumonia-like organism isolated by Herick & Eaton (1945) from embryonated eggs. Reticulum-cell hyperplasia and a polymorphonuclear exudate were produced in mice by strains which caused pneumonia (Edward, 1940). Other strains from rats and mice were pyogenic and produced local abscesses. Some members of the group show marked affinities for certain tissues; a tendency of most species to localize in joints has attracted considerable attention, but there is no evidence that rheumatism in man is caused by pleuropneumonia-like organisms (Findlay, 1946).

DETAILS OF INDIVIDUAL SPECIES

Preliminary remarks

Although a number of different species have been described, many were identified by serological and pathogenic properties alone; sometimes only morphology and cultural appearances were studied. These descriptions are not adequate for comparisons with recently isolated strains to be made. Since many of the original strains were lost, while antisera prepared against them are no longer available, re-examination is impossible. Examination of strains representing as many species as could be collected showed that most species could be recognized by characteristic, cultural and biochemical properties (Edward, 1950*b*, and unpublished observations). Numerous freshly isolated strains of some species were examined, but with other species only one, or a few, laboratory-adapted strains were available for study and thus their properties may not be completely characteristic of the given species.

All pleuropneumonia-like organisms isolated should be identified when possible according to species. To allow comparisons to be made with the recognized species a collection of freeze-dried cultures of representative strains is maintained at the Wellcome Research Laboratories, Beckenham, Kent together with a number of antisera, also preserved by freeze-drying. Contamination of one organism by another is a constant hazard in bacteriology, but with bacteria this is usually quickly and easily recognized. Unfortunately, when strains of pleuropneumonia-like organisms become mixed, in spite of precautions, this may not be readily apparent because both strains may have identical colonial appearances. Thus the importance of knowing those properties which can be used to check the identity of a particular strain needs no emphasis.

The few cultural and biochemical tests which are at present available for studying strains, are as follows:

(i) *Growth in a semi-solid medium.* Some strains grow equally well throughout a semi-solid medium, others which prefer aerobic conditions grow best near the surface, and a few strains grow better near the bottom than near the surface. The growth may be smooth and fluffy, or granular. Sometimes a strain is neither definitely smooth nor definitely granular.

(ii) *Growth on rabbit serum agar.* Some strains grow poorly on a medium enriched with rabbit serum; others grow as well on a rabbit serum medium as they do on a medium containing horse serum.

(iii) *Formation of a film and spots.* These are noted by growing the strain on horse serum agar for 6 days.

(iv) *Fermentation of carbohydrates.* Some strains ferment certain carbohydrates. Lactose, sucrose, mannitol, dulcitol, sorbitol, rhamnose, xylose, trehalose, raffinose, inositol, inulin and salicin were not fermented by any strain tested; dextrin, starch and glycogen were fermented by those strains which fermented glucose.

(v) *Haemolysis.* Strains produce a variable degree of haemolysis of horse blood agar. The filtrates of 6-day broth cultures of a few strains are capable of discolouring suspensions of horse erythrocytes. Table 1 shows how these tests assist the identification of species.

The numerous organisms which have been described can be conveniently grouped according to habitat, but this arrangement is only provisional. Sabin (1941*a*, *b*) classified and named species according to the animal host, but physiological properties, when more becomes known about them, seem likely to provide a more suitable means of classification, as with bacteria. It is already apparent that there is a difference between those species which ferment carbohydrates and those which do not.

Organisms from cattle

Asterococcus mycoides, the type species, causes contagious pleuropneumonia. It grew poorly on rabbit serum agar and did not form a film and spots on horse serum agar (Table 1). In a semi-solid medium it grew best near the surface, producing a smooth growth (Edward, 1950*b*). When a broth culture was shaken the sedimented growth rose in characteristic silky swirls (Walker, 1930). It fermented glucose, fructose, maltose, mannose, dextrin, starch and glycogen, but not galactose; slight fermentation of sucrose and trehalose has also been reported (Tang *et al.* 1935). Indole was not formed. The organism produced haemolysis on horse blood agar and differed from all other organisms of the group except the organism causing pleuropneumonia of goats in that discoloration of horse erythrocytes was produced by filtrates of 6-day fluid cultures (Warren, 1942; Edward, 1950*b*). It also liquefied inspissated serum (Edward, unpublished). For reasons stated later (p. 48), it is suggested that this organism should be known as *A. mycoides*.

P strains. Organisms of a different species have been isolated from the bovine genital tract (Edward, Hancock & Hignett, 1947). All strains which required serum for growth produced a film and spots on horse serum agar and were regarded as a single species, provisionally designated by the letter P (Edward, 1950*a*, *b*). The serological examination of ten strains showed that they shared common antigens. The organisms grew equally well throughout a semi-solid medium, giving a smooth growth (Table 1). They grew only poorly on rabbit serum agar. They produced haemolysis of horse blood agar but did not ferment carbohydrates. It is possible that P strains may cause inflammation of the genital tract, predisposing to infertility, but the experimental inoculation of heifers *in utero* with cultures was without effect (Edward, 1952).

S strains. Occasionally strains capable of growth on serum-free media were isolated from samples taken from the genital tract (Edward, 1950*a*). These S strains were probably not inhabitants of the genital tract but reached samples as contaminants; they are included among the saprophytic strains.

Organisms from sheep and goats

Organism of contagious agalactia of sheep and goats. Although this organism, named *Anulomyces agalaxiae* by Wroblewski (1931), was first isolated in 1923 by Bridré & Donatien, who pointed out its similarity to the organism of contagious bovine pleuropneumonia, there have been few attempts to study its cultural and biochemical properties for comparison with those of the organism of bovine pleuropneumonia. In continental literature it is still the practice to call the organism of agalactia a virus (Zavagli, 1951), and this fact may possibly account for the failure to study its cultural properties adequately. In view of the importance of the organism in veterinary medicine it was therefore necessary for this review to collect strains for study. Five strains were obtained through the kindness of Prof. P. Hauduroy from Dr Anguelov of Sofia, from Dr C. Lopez of Madrid and from Prof. V. Zavagli of Rome. Four of the five strains produced a film and spots on horse serum agar; a film containing lipids on the surface of fluid cultures has been described previously (Pirie & Holmes, 1933; Nowak & Lominski, 1934). All the strains grew best near the surface of a semi-solid medium producing a smooth growth; they only grew poorly on rabbit serum agar (Table 1). They produced haemolysis on horse blood agar, but did not ferment carbohydrates (Edward, 1953*b*).

In addition to these five strains of *A. agalaxiae* a sixth strain, also believed to be the organism of agalactia, was received. The properties of this strain, however, were different from those of the other six strains and were identical with those of organism of goat pleuropneumonia (see below). This emphasizes the necessity to examine every strain of pleuropneumonia-like organism sufficiently for its species to be identified; the identification of strains from goats is particularly important, because two species of the pleuropneumonia group are pathogenic for this animal.

Organism of contagious pleuropneumonia of goats. It was only recently recognized that contagious pleuropneumonia of goats was caused by an organism of the pleuropneumonia group. This disease has been reported from many parts of Europe, Asia and Africa. It is of considerable economic importance as it is almost always fatal; in Nigeria alone it is estimated to cause losses of £70,000 per annum. The infective agent was believed to be a filterable virus until Longley (1951), whose work was confirmed independently by Beveridge & Chu (personal communication), isolated a pleuropneumonia-like organism and reproduced the disease with cultures. Lesions were produced in sheep by experimental inoculation, but the sheep did not acquire the disease under natural conditions. The organism was serologically distinct from the organism causing bovine pleuropneumonia.

Well-separated colonies of two strains, one isolated by Longley and the other by Chu, reached a diameter of 1·5 mm. after 3 days of incubation and were thus noticeably larger than those of any other organism of the group. Chu's strain grew best near the surface of a semi-solid medium, producing a smooth growth; it grew well on rabbit serum agar and did not form a film and spots (Table 1). There was slight growth on the basal medium alone without serum enrichment. The organism liquefied inspissated serum and fermented glucose, fructose, maltose and mannose, but not galactose. It produced haemolysis of horse blood agar. The supernatant from a 6-day fluid culture discoloured horse erythrocytes and it was the only strain, apart from the organism causing bovine pleuropneumonia, which showed this property (Edward, 1953*b*).

Pleuropneumonia of goats resembles bovine pleuropneumonia in symptomatology and pathology; the causative organisms have similar properties and the only significant differences noted between them were in host and in antigenic constitution. It is possible that the disease in goats is caused by a bovine organism which has become adapted to goats. Under experimental conditions goats were infected with cultures of the bovine organism, grown in media containing sheep serum (Dujardin-Beaumetz, 1906). The susceptibility of cattle to the goat organism does not appear to have been tested experimentally, but cattle do not acquire the disease from infected goats under natural conditions. It is therefore suggested that the organism of goat pleuropneumonia should not be assigned to a new species, but should provisionally be regarded as a new variety, to be called *Asterococcus mycoides* var. *capri*.

Melanidi (1951) drew attention to an infective oedema and cellulitis which was endemic among goats around Sparta in Greece and was usually fatal. The disease was regarded as a hyper-acute type of contagious agalactia, because the causative organism, a member of the pleuropneumonia group, appeared to be serologically the same as the organism of contagious agalactia. It is, however, apparent that this naturally occurring infection produces a condition similar to that produced experimentally by the subcutaneous inoculation of the organism of goat pneumonia. A strain of the organism, isolated from an infected goat in Sparta and kindly provided by Dr Melanidi, had the cultural and biochemical properties of the organism of goat pneumonia and not those of the organism of agalactia (Edward, 1953*b*). Further investigation should therefore be made into the aetiology of the disease in Sparta, and it would seem that the disease is more likely to be caused by the organism responsible for pleuropneumonia in goats than by the organism of agalactia. The value of examining strains of organisms by cultural and biochemical methods, as well as by serological methods, must be emphasized.

Other strains from sheep. Pleuropneumonia-like organisms were isolated from the feet of sheep affected by foot rot (Beveridge, 1941). Cultures of the organisms did not reproduce the disease and properties of the strains were not studied further.

Organisms from dogs

Shoetensack (1934, 1936*a*, *b*) isolated organisms of the pleuropneumonia group from the tissues and nasal secretions of dogs suffering from distemper. The strains belonged to two types, *Asterococcus canis* types I and II, which differed from each other serologically (Klieneberger, 1938). Their pathogenicity was uncertain. *A. canis* type I was believed by Shoetensack to be the cause of the distemper, *A. canis* type II being possibly a secondary invader. Since secondary infection is an important feature of the disease caused by distemper virus, it is possible that both types of organism were secondary invaders. These organisms of Shoetensack cannot now be compared with others more recently isolated from dogs, because cultures appear to be no longer in existence, and the original descriptions were inadequate.

Edward & Fitzgerald (1951*a*) found pleuropneumonia-like organisms in a high proportion of cultures from throat and vagina of bitches. In some cultures there were three different types of colony and by subcultivating each type separately organisms with different colonial and serological properties were obtained. Most of the strains belonged to three species, provisionally designated as α, β and γ; a few strains which appeared different were not studied further. None of these species is known to be pathogenic, but the α strains merit further investigation for possible pathogenicity.

The α strains, isolated from vagina and semen, formed coarsely reticulated colonies; the centre appeared to contain large globules which tended to obscure the central spot, while the periphery was transparent. These colonial characteristics were lost on repeated subculture. The organisms grew poorly on rabbit serum agar and did not form a film and spots on horse serum agar (Table 1). They grew throughout semi-solid media, but their growth was neither definitely granular nor completely smooth. Glucose was not fermented and horse blood agar was only feebly haemolysed.

The β strains, isolated from vagina and throat, formed relatively large flattened colonies with small poorly developed central spots. These characteristic appearances were quickly lost by subcultivation. The strains differed from the α strains by growing well on rabbit serum agar and by giving more marked haemolysis; they were also serologically different (Table 1).

The γ strains, also isolated from vagina and throat, produced a film and spots on horse serum agar; their colonies were not otherwise distinctive. They grew only poorly on rabbit serum agar, produced haemolysis of blood agar and did not ferment glucose (Table 1).

Organisms from rats

L3 organism. Numerous isolations of a pleuropneumonia-like organism, known as the L3 organism, were made by Klieneberger & Steabben (1937, 1940) from the lungs of laboratory rats; most of the animals had bronchiectasis but some were without lung lesions. Once the organism was isolated from a wild rat. Four strains from laboratory rats and the one strain from a wild rat were serologically similar (Klieneberger, 1938). The association of the organisms with bronchiectasis suggested that they were pathogenic, but the inoculation of cultures into rats was without effect, although abscesses were produced locally in mice by injecting cultures subcutaneously together with agar.

The L3 organism differed from other strains in its colonial appearances. The surface of a colony was somewhat rough and the surface markings tended to obscure the central spot. When one of the original strains was re-examined, it produced a film and spots on horse serum agar, grew poorly on rabbit serum and grew better near the surface of a semi-solid medium, the growth being granular (Table 1). It haemolysed horse blood and fermented glucose, maltose, and mannose, but not fructose or galactose (Edward, 1950*b*). Unfortunately this strain was lost. Dr E. Klieneberger-Nobel kindly provided another strain which she had recently isolated from the lung of a rat. The new strain differed from the original in several respects. It did not have the same colonial appearances; it grew equally throughout a semi-solid medium, did not form a film and spots and fermented fructose, feebly, but not mannose. Moreover, it was not pathogenic for mice on subcutaneous inoculation. Possibly more than one species inhabit the lungs of rats. The L3 organism has also been found in mice. Klieneberger & Steabben (1940) isolated one strain from the brain of a mouse and a strain isolated by Edward from the nose of a mouse with infectious catarrh was serologically identical with the L3 organism (Klieneberger & Smiles, 1942). The similarity between the L3 organism and strains isolated from mice with infectious catarrh is discussed on p. 52.

The L4 organism. The antigenically different L4 organism was originally isolated by Klieneberger (1938) from the submaxillary gland of a rat; it was serologically identical with a pyogenic agent which contaminated a transmissible sarcoma (Klieneberger, 1939) and with a pleuropneumonia-like organism which caused polyarthritis in rats (Findlay *et al.* 1939*b*). It produced abscesses when inoculated into rats and mice; in rats there was a tendency to localize in the joints. Intracerebral injection of mice caused encephalitis.

Pleuropneumonia-like organisms with similar properties were isolated by other workers from wild and laboratory rats with polyarthritis, but their identity with the original L4 strain was not checked serologically (Beeuwkes & Collier, 1942; Preston, 1942). The strain isolated by Preston grew well on rabbit serum agar, did not form a film and spots, gave a smooth growth throughout a semisolid medium and haemolysed blood, but did not ferment carbohydrates (Table 1) (Edward, 1950*b*).

Organisms from mice

Many different strains of pleuropneumonia-like organisms have been isolated from mice, and their relationship to each other is confusing; possibly some are identical with organisms found in the lungs of rats.

Sabin's strains. Sabin (1941*a*) isolated five serologically different types of organism from nose, conjunctival sac, respiratory tract and even brain of normal mice. Sometimes three different types of organism were isolated from the same culture (Sabin & Johnson, 1940*b*). Intravenous inoculation of mice with types, A, B, C, D and E produced arthritis. Type A was also capable of multiplication in the brain and caused encephalitis after intracerebral inoculation. The affected mice exhibited peculiar nervous signs known as 'rolling disease'. Sabin's strains no longer exist for comparison with other strains.

The L5 organism, serologically identical with Sabin's type A, was isolated by Findlay and his colleagues (1938) from the brains of mice used for passaging lymphocytic choriomeningitis virus. It differed from Sabin's strain in being less virulent and in not forming exotoxin. A strain of L5, which had lost its virulence after several years in subculture, grew poorly on rabbit serum agar, did not form a film and spots, haemolysed blood and fermented glucose, maltose and mannose, but not fructose or galactose (Table 1) (Edward, 1950*b*). It gave a granular growth, best near the surface of a semi-solid medium, whereas its growth originally had been smoother (Klieneberger, 1940).

The L6 *organism* was isolated from the brains of mice which had been inoculated with mouse blood containing *Eperythrozoon coccoides* (Findlay *et al.* 1939*a*). Its colonies, larger than those of L5, were coarsely marked and vacuolated; growth in broth was granular. It differed from the L5 organism serologically (Klieneberger, 1940).

From infectious catarrh. Pleuropneumonia-like organisms were found associated with infectious catarrh, a chronic infection of the respiratory tract of mice, frequently complicated by otitis media and pneumonia. The disease is endemic in some stocks of laboratory mice and intranasal inoculation of infected mice caused pneumonia which was transmissible in series (Edward, 1940). Nelson (1937), who at first failed to isolate pleuropneumonia-like organisms from lesions in this disease, ascribed the infection to 'cocco-bacilliform bodies', visible inside pus cells. These bodies were grown in tissue culture. Later, he agreed that pleuropneumonia-like organisms were associated with the disease (Nelson, 1948). Early subcultures of the organisms reproduced the disease and cocco-bacilliform bodies were present in the lesions (Edward, 1947*a*).

Strains isolated from mice with catarrh differed in their colonial appearances. The colonies of some strains had the appearance typical of organisms of this group; the colonies of other strains resembled those of the L3 organism and had a rougher surface. Recently pleuropneumonia-like organisms were isolated on numerous occasions from a strain of infectious catarrh, maintained in mice by serial passage (Edward, unpublished observations). Primary isolation was possible on solid media, although this had not been accomplished in earlier work. Growth in the primary culture was improved by the addition of thymus nucleic acid to the medium. All the strains had the same properties; their colonies were of the rough type. They grew well on rabbit serum agar, formed a film and spots on horse serum agar, haemolysed blood and fermented glucose, maltose and mannose; fermentation of fructose was weak and variable and there was possibly slight fermentation of galactose (Table 1). In a semi-solid medium growth was better near the surface; growth of a freshly isolated strain was smooth, but it became granular during subcultivation. Previously reported findings must be disregarded as the strain examined was later found not to have originated from a mouse (Edward, 1950*b*).

Although the strains lost their ability to produce pneumonia after a few subcultures, they produced abscesses even after many subcultures when inoculated subcutaneously into mice together with agar. Cocco-bacilliform bodies were not visible in the pus from these abscesses. The organisms thus resemble the L3 organism in pathogenic and cultural properties, and it is possible that the same organism inhabits both mice and rats. Possibly organisms of different species were found in cultures from mice belonging to different stocks. An antiserum prepared against one strain failed to agglutinate two of five other strains (Edward, 1940).

From conjunctivitis. Pleuropneumonia-like organisms were found associated with endemic conjunctivitis (Nelson, 1950*a*, *b*). Their colonies differed from those of strains isolated from mice with catarrh. Cultures did not cause conjunctivitis unless very heavy suspensions were instilled into the eye.

Organisms from fowls

A pneumonia virus which was being passaged in embryonated eggs was found to be contaminated with a pleuropneumonia-like organism. Cultures of the organism produced haemagglutination, which was inhibited by an antiserum prepared in a rabbit and by sera from a high proportion of hens in the hatchery providing the eggs. It thus seemed likely that the strain had been picked up from the eggs (Herick & Eaton, 1945).

An infective agent has been isolated from cases of upper respiratory catarrh in fowls; after passage in embryonated eggs it reproduced the disease in fowls. A similar agent was isolated from turkey sinusitis. Both agents were visible microscopically in infected tissues and were regarded as viruses (Van Roekel, Olesiuk & Peck, 1952). Markham & Wong (1952) isolated pleuropneumonia-like organisms from egg-passage material containing each of the agents. After thirteen successive subcultures in artificial media the organisms produced specific lesions in chick embryos. Suspensions of the tissues of these embryos produced catarrh in fowls. Infections of the upper respiratory tract of fowls and turkeys are of considerable economic importance, and further work is needed to establish the significance of the pleuropneumonia-like organisms and their relationship to the viruses and cocco-bacilliform bodies which have been found associated with these infections (Nelson, 1935). Pleuropneumonia-like organisms have been isolated from the upper respiratory tract of apparently healthy fowls, and even strains isolated from birds with catarrh have not produced lesions after experimental inoculation (Beveridge & Chu, personal communication).

A strain of pleuropneumonia-like organism isolated from the upper respiratory tract of a fowl was received from Dr H. P. Chu. The organism formed a film and spots on horse serum agar, grew well on rabbit serum agar and grew throughout a semi-solid medium as a smooth growth (Table 1). It haemolysed blood but did not ferment glucose (Edward, unpublished observations).

Organisms from man

Pleuropneumonia-like organisms have been shown to be frequent inhabitants of the human genital tract and anal canal (Dienes, 1940; Dienes *et al.* 1948; Harkness, 1950). They were isolated from the urethra of about 20% of cases of non-specific urethritis in man. As urethral cultures from healthy males were at first negative, it seemed possible that the organisms were a cause of non-specific urethritis (Beveridge, Campbell & Lind, 1946; Harkness & Henderson-Begg, 1948). However, in two more recent investigations the organisms were isolated nearly as frequently from healthy males as from patients with urethritis (Harkness, 1950; Melén & Linnross, 1952). Even more frequent isolations were made from cervix and vagina of women; the proportion of positive isolations was significantly greater in those with inflammation of the genital organs than in those without inflammatory lesions (Klieneberger-Nobel,1945; Randall, Stein & Ayres, 1950; Melén & Odeblad, 1951, 1952).

The organisms appear to be transmitted usually by sexual intercourse, but their role in causing inflammation of the genital tract is doubtful (Edward, 1952). A recent investigation tends to confirm the view that the pleuropneumonia-like organisms are not the cause of non-specific urethritis (Nicol & Edward, 1953). This disease in patients with positive cultures for pleuropneumonia-like organisms did not differ in its clinical course or in its response to treatment from the disease in those with negative cultures; there is therefore no evidence for regarding the organisms as important secondary invaders. Although the organisms appear to be commensals, the possibility cannot be excluded that they may cause suppurative lesions under special circumstances.

A search has been made for pleuropneumonia-like organisms in joint fluid from cases of arthritis which had occurred as a complication of non-specific urethritis. Positive cultures were obtained from a few cases, but the cultures were more often negative (Dienes *et al.* 1948; Kuzell & Mankle, 1950). The significance of the positive findings is doubtful, because the isolations of pleuropneumonia-like organisms from brains of healthy mice suggest that organisms of the pleuropneumonia group are able to remain latent inside the body.

A total of ninety-one strains of pleuropneumonia-like organisms, isolated in London from genital tract and anal canal, had, with one exception, the same biological properties and were serologically related to each other (Nicol & Edward, 1953). Two strains isolated in Paris and two of five strains from the U.S.A. were also similar. These strains will be referred to as human type 1. They grew well on rabbit serum agar and did not form a film and spots (Table 1). Growth, which was usually markedly granular, occurred throughout semi-solid media. They did not ferment glucose and produced little or no haemolysis of horse blood agar. All the strains were related to each other serologically, although they reacted differently with antisera prepared against four of them. One strain, H. 23, was not agglutinated by antisera to three other strains, yet an H. 23 antiserum agglutinated to full titre strain H. 26, which itself was agglutinated by the other three antisera. Strains isolated from persons with no evidence of genital infection did not differ in their serological or other properties from strains isolated from patients with genital infection; strains from rectum and anal canal were similar to those from cervix and urethra.

A few strains were isolated on media incubated anaerobically, the corresponding cultures on media incubated aerobically being negative. The strains grew well on media at pH values between 7·0 and 8·0 when the plates were incubated anaerobically or in an atmosphere containing 10 % CO_2, there being little or no growth under aerobic conditions. Good growth, however, was obtained aerobically on media adjusted to pH 6·5 and 6·0. These strains did not differ from the usual type 1 strains in their serological or other properties.

Five strains isolated in the U.S.A. were received from Prof. H. E. Morton; three of them did not react with any of the type 1 antisera, but resembled each other serologically. They also differed from type 1 strains, because abscesses were produced locally when cultures containing agar were injected subcutaneously into mice. Their biochemical properties were the same as those of type 1 (Table 1). They will be provisionally called human type 2. One of the three strains, 'Campo L', was originally isolated by Dr Dienes and found to be serologically similar to five other strains isolated by him in 1939–40 (Dienes, personal communication). Four other strains isolated in America were shown to be identical serologically with 'Campo L' (Norman, Saslaw & Kuhn, 1950).

Pleuropneumonia-like organisms, which appeared to differ from the type usually isolated from the genital tract, were cultured by Ruiter & Wentholt (1952) from four patients with ulcerative lesions of the glans penis; they were called 'G-strains'. They were associated with fusiform bacilli and other bacteria, so that their pathogenic significance was uncertain. They were difficult to grow and needed anaerobic or microaerophilic conditions. Abscesses were produced when cultures were inoculated into the foot pads of mice. A strain which had lost its virulence for mice was kindly provided by Prof. M. Ruiter. Its growth was improved by adding thymus nucleic acid to the medium, when it became as good as the growth of type 1 strains provided the cultures were incubated anaerobically. Under aerobic conditions there was only a trace of growth on media adjusted to pH 7·5–8·0, but there was moderately good growth on media at pH 6·5. The strain also grew moderately well in an atmosphere containing 10 % CO_2. A film and spots were formed on horse serum agar; growth on rabbit serum agar was as good as on horse serum agar (Table 1). In a semi-solid medium growth which was smooth was found near the bottom of the tube. Glucose, fructose and maltose were fermented, but not mannose or galactose. Only a trace of haemolysis was produced (Edward, unpublished observations). This strain will be designated as human type 3.

All the ninety-one strains isolated in this laboratory from the genital tract were identified as type 1 with the exception of one strain, H. 106. This strain differed serologically from types 1, 2 and 3, but resembled type 3 in fermenting the same carbohydrates and in forming a film and spots (Table 1). It grew well anaerobically without adding thymus nucleic acid to the medium, but under aerobic conditions its growth was negligible.

Pleuropneumonia-like organisms also frequently inhabit the mouth and pharynx. Morton and his colleagues (Morton *et al.* 1951, 1952; Smith & Morton, 1951*b*) isolated strains on media enriched with horse serum incubated aerobically, but in this laboratory successful isolations were only obtained under anaerobic conditions. Growth was better on rabbit serum agar than on horse serum agar and was improved by adding thymus nucleic acid to the medium. When the thymus nucleic acid was not added growth often took place only near bacterial colonies; it is possible that the failure to subculture the 'X' colonies noted by Sabin & Johnson (1940*a*) was due to absence of thymus nucleic acid from the medium.

Three of five strains isolated from saliva formed a film and spots. Although two strains did not exhibit this property, in other respects the five strains behaved similarly. They gave a smooth growth at the bottom of semi-solid media; they did not haemolyse horse blood and did not ferment glucose (Table 1). An antiserum prepared against one strain inhibited the growth of each of the five strains, although it failed to agglutinate the homologous organism to a significant titre. Growth was not inhibited by antisera to the types of organism isolated from the genital tract (Edward, unpublished observations). These strains will be called Human Type 4. Dienes & Madoff (1953) showed that the strains they isolated from the mouth differed serologically and in colonial appearances from the genital strains. Their strains, like those of Morton and his colleagues, were isolated under aerobic conditions without adding thymus nucleic acid to the medium. It is not yet clear what relationship the American strains bear to those isolated in this laboratory, which require different cultural conditions.

Paine and his colleagues (1950) reported the isolation of a pleuropneumonia-like organism from a patient with meningitis and a brain abscess, which had resulted from a tobacco pipe being thrust into the brain through the orbital wall. A streptococcus was also present in the pus, so that the infection could not with certainty be ascribed to the pleuropneumonia-like organism. The pleuropneumonia-like organism did not grow anaerobically; haemolysis and fermentation of carbohydrates were not detected.

Pleuropneumonia-like organisms were also isolated from the blood of another patient with a brain abscess and from the blood of three children with purpura (Carlson *et al.* 1951). It is interesting that one of the infections followed a human bite. The strains, which are no longer available, were not sufficiently studied for their relationship to the genital and mouth strains to be established. Although they were not proved to be responsible for the infections, these reports suggest the wisdom of looking for organisms of the pleuropneumonia group in infections of unknown aetiology.

Saprophytic strains

These are capable of growing on serum-free media without the addition of cholesterol. The optimum temperature for growth is 30°, but moderate growth occurs at 37 and 22°. They were first isolated from sewage by Laidlaw & Elford (1936), and from decomposing vegetable matter by Seiffert (1937*a*, *b*). Similar strains were also encountered as contaminants in cultures made from the genital tract of cattle (Edward, 1950*a*). The sewage A and B strains of Laidlaw & Elford, although differing somewhat serologically, shared common antigens; Seiffert's strains were closely related to sewage A (Klieneberger, 1940). S strains encountered in cultures from the bovine genital tract were also serologically related to sewage A and B. An antiserum prepared against one S strain fixed complement with suspensions of sewage A and B, although it did not agglutinate them. One of Laidlaw & Elford's strains, sewage C, was antigenically distinct from sewage A and B, and differed in its nutritional requirements by requiring haemin (Pirie, 1937, 1938; Holmes, 1937). It is unfortunate that this strain, which was of a different type from the others, appears to have been lost.

Sewage A and B strains did not form a film and spots; they grew best near the surface of semi-solid media (Table 1). Horse blood was haemolysed and the strains fermented glucose, fructose and maltose, but not galactose or mannose. An S strain gave the same reactions, except that it fermented galactose (Edward, 1950*b*).

CONCLUSION

The nature of the pleuropneumonia group of organisms must be considered in the light of their properties. They have on the one hand been classed as viruses, and on the other have been included among the bacteria. Like bacteria, they can be grown on cell-free media; most strains appear to need cholesterol or another sterol for growth, whereas cholesterol is not known to be an essential nutrient for any bacteria. Some strains when first isolated from animals appear to be defective in synthesizing deoxyribonucleic acid; bacteria synthesize their own nucleic acids and the most exacting require to be given only certain purines, pyrimidines and nucleosides, but viruses depend in this and other respects on the synthetic mechanisms of the cells they parasitize. The pleuropneumonia-like organisms have distinctive morphological and colonial appearances, but some bacteria form a variant or L-phase which resembles a pleuropneumonia-like organism in morphology and colonial appearance. In addition to division by binary fission the pleuropneumonia-like organisms appear to be capable of multiplication by multipolar germination in a manner somewhat similar to the intracellular multiplication of certain viruses. An understanding of the mode of reproduction of viruses may possibly be obtained by studying the growth and multiplication of organisms of the pleuropneumonia group, because there appear to be certain similarities between the two methods.

Cultures of organisms of the pleuropneumonia group contain elements as small as the particles of some true viruses. Pleuropneumonia-like organisms also exhibit other similarities to viruses. The injection of hyperimmune serum into an animal before inoculating it with a pathogenic member of the pleuropneumonia group will prevent infection. Hyperimmune sera are of little use in preventing bacterial infections, whereas most viruses can be neutralized both *in vitro* and *in vivo*. Growth of a pleuropneumonia-like organism is prevented by incorporating an antiserum in the medium; this phenomenon has certain similarities to the neutralization of a virus. Growth of bacteria in the L-phase is inhibited by antiserum, but bacteria in the bacillary phase do not appear to be inhibited.

The evidence thus appears to support the opinion of Sabin (1941*a*) that the pleuropneumonia group of organisms belong to a class distinct both from bacteria and viruses, although they have properties in common with both these classes. They appear to be particularly closely related to bacteria through the L-phase variants of the latter. Dienes & Weinberger (1951) suggested that pleuropneumonia-like organisms might have evolved from bacteria by stabilization of L-phase variants, but, since the bacillary to L-phase variation has been shown to be reversible with many bacteria, it could be suggested that bacteria were derived from pleuropneumonia-like organisms which now represent a persistence of a form of micro-organism found early in evolution.

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(*Received* 3 *June* 1953)

ADDENDUM

Moustardier, Brisov & Perry (1953) have claimed that 4 of 13 strains of pleuropneumonia-like organisms, isolated from the human genital tract, when subcultured on ordinary media, changed into bacteria, two strains into *Streptococcus faecalis*, one into *Proteus mirabilis* and one into *Bacterium faecalis alkaligenes*. These findings were adduced as evidence that the genital pleuropneumonia-like organisms are the stabilized L-phase of various bacteria and it was suggested that the strains which did not revert were possibly the L-phase of the gonococcus. In view of the significance of these claims, which are difficult to reconcile with the findings of Nicol & Edward (1953) that all except one of 91 strains of pleuropneumonia-like organisms, isolated from the genital tract, were serologically similar, it is unfortunate that the authors give few experimental details. The biological and serological properties of the pleuropneumonia-like organisms were apparently not examined for comparison with those of the bacteria into which the organisms appeared to change. Moreover, it is impossible to determine from the report whether the original colonies were typical of organisms of the pleuropneumonia group or had the colonial characteristics shown by L-phase organisms.

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Debaryomyces marama n.sp., Isolated from the Air

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SUMMARY: A new species of *Debaryomyces* has been isolated from the air. It is characterized by a four-spored ascus, the production of a pellicle, lack of pseudomycelium, and the ability to assimilate glucose, sucrose, maltose and galactose but not lactose.* It has been named *Debaryomyces marama.*

In the course of a survey of aerial yeasts, made with a Manning slit sampler, fifteen strains of a hitherto undescribed species of *Debaryomyces* were isolated. It is proposed to name this species *D. marama*, from the Maori adjective 'marama' meaning 'light, not dark'.

Debaryomyces marama sp.nov.

Cellulae in culturis novis rotundae aut subovoidae, singulae aut in racemis parvis, gemmantes multifarium $(2{\cdot}5-6{\cdot}5)\times(2{\cdot}5-5{\cdot}0)\ \mu$. Pseudomycelium nullum observatum est. Pellicula tenuis sed tenax et arida, sursum repens per latera vasculorum in glucoso cum decocto saccharomycetis (minus biduo 27°). Culturae novae in agaro Sabouraudi abundantes, albae, aliae nitidiores, aliae hebetiores, saepe aetate fiunt flavalbidae et subcrispulatae. Copulatio cellularum iniquarum conformationi asci praecedit. Ascosporae rotundae aut subovoidae, glabrae, 1–4 in asco. In ascis tetrasporis sporae in tetrahedram formantur. Interdum fermentatio iniqua glucosi. Glucosum, sucrosum, maltosum, galactosum assimilantur. Lactosum, nitras kalicus non assimilantur. Non crescit (37°).

Cells from young cultures round to slightly oval, single or in small clusters. Budding multipolar. Cell size $(2{\cdot}5-6{\cdot}5)\times(2{\cdot}5-5{\cdot}0)\ \mu$. No pseudomycelium observed. A light but persistent dry pellicle which spreads up the sides of the tubes in glucose yeast extract broth held at 27° for less than 2 days. Young cultures on Sabouraud agar abundant, white and glistening to matt, often becoming light fawn and wrinkled with age. Heterogamous conjugation precedes ascus formation. Spores round to slightly oval, smooth, and one to four per ascus. In four-spored asci the spores arranged in tetrahedra. Glucose occasionally weakly fermented. Glucose, sucrose, maltose and galactose assimilated; lactose and potassium nitrate not assimilated. No growth at 37°.

DISCUSSION

This yeast is included in the genus *Debaryomyces* as emended by Lodder & van Rij (1952) (at present a 'nomen conservandum' because of these authors' proposition that the type species, the fermentative *D. globosus*, be excluded).

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* Miss W. Sloof of the Yeast division, Centraalbureau voor Schimmelcultures, reports that the strain deposited there assimilated lactose when vitamins were added to the auxanographic medium. In the tests made in this laboratory growth factors were supplied to the medium in the form of a drop of a solution of Difco yeast extract.

The essential characters in their definition of the genus are as follows. Cells round to short oval. Vegetative reproduction by multilateral budding. A primitive pseudomycelium may be formed. Generally heterogamous conjugation precedes ascus formation. Spores round, usually one per ascus, seldom two, exceptionally up to four (see Fig. 1). A dull, dry, creeping pellicle may be formed. No fermentation or very weak fermentation. Nitrate not assimilated.

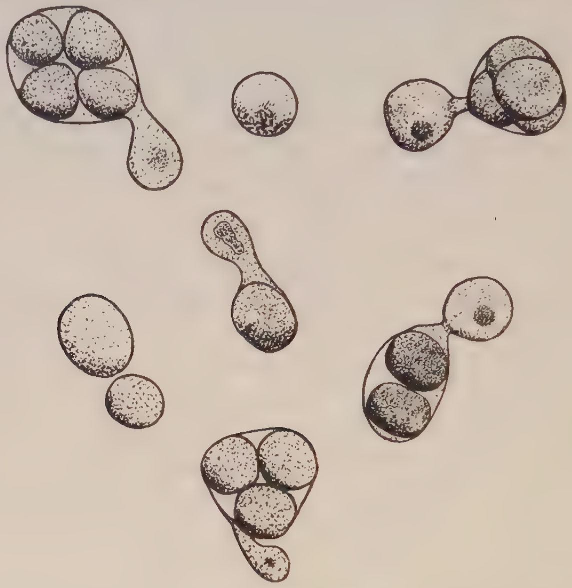

Fig. 1. Asci and ascospores of *Debaryomyces marama*. From Gorodkowa agar.

D. marama differs from *D. vini*, hitherto the only four-spored Debaryomyces described, in its lack of pseudomycelium and its ability to produce a rapidly formed pellicle, whilst resembling it in the sugars which it can assimilate. Presence or absence of a pseudomycelium of a primitive type is not, in the yeasts, a sufficiently reliable character upon which to separate species, but ability or lack of ability to form a pellicle is, and has been used as the distinction between other *Debaryomyces* species (*D. hansenii* and *D. subglobosus; D. nicotianae* and *D. kloeckeri*).

Lodder & van Rij consider that *D. vini* is intermediate between the genera *Debaryomyces* and *Pichia*, resembling *Pichia* in its four-spored asci and pseudomycelium, but lacking a pellicle so that its inclusion in that genus is impossible. By this example, therefore, it would seem that *D. marama* should be placed in *Pichia*. Nevertheless, I feel that it would be improper to do so. Its resemblance to *D. vini*; its lack of pseudomycelium; the shape of its cells, typical of Debaryomyces and not the long-oval of Pichia, place it in the Debaryomyces. Ecologically also its distribution puts it with *Debaryomyces*, for all the species of this genus and none of *Pichia* have been found in the course of the survey on aerial yeasts.

A strain of this yeast has been dispatched to the Centraalbureau voor Schimmelcultures (Yeast Division), Delft. Strains are also being sent to the Institute of Brewing Yeast Collection, Nutfield, Surrey, and to the American Type Culture Collection.

I wish to thank Prof. G. R. Manton, Classics Department, Otago University, for assistance with the preparation of the Latin diagnosis.

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(*Received* 12 *June* 1953)

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A New Type of Polar Monotrichous Flagellation

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SUMMARY: An organism with a new type of polar monotrichous flagella is described. The flagella have a very short wavelength which is very characteristic. The organism liquefies gelatin and oxidizes ethanol to acid. All other common differential tests were negative. The name *Pseudomonas diminuta* n.sp. is suggested.

The curvature of bacterial flagella is quite constant and sufficiently characteristic to be of considerable taxonomic importance. The usual wavelength of the flagella of such polar monotrichous bacteria as *Pseudomonas aeruginosa* and *Vibrio comma* is in the neighbourhood of 2 μ. In a large collection of cultures of Gram-negative bacteria submitted to the authors for flagellar study by Robert Keller of the Museum of Natural History of Philadelphia were found three cultures which showed very unusual monotrichous flagella, having a wavelength of only slightly over 0·6 μ. The cultures originated from fresh-water streams. Morphologically and physiologically the three cultures were identical and one description will apply to all.

MORPHOLOGY OF ORGANISM

The organism is a small Gram-negative rod averaging 0·5 by 1·0 μ in the usual peptone media. Capsule or spore formation was not demonstrable. It is actively motile with predominantly a single polar flagellum. A flagellum at each pole is fairly common in young cultures, and occasionally two flagella may be found on one pole. The flagella have two unusual characteristics: (1) Many of the individuals show the flagellum originating from the periphery of the pole rather than from the centre of the pole, the latter being the more usual origin with monotrichous bacteria. (2) The flagellar curvature is most unusual with an average wavelength of only 0·62 μ, and an average wave amplitude of 0·32 μ. The standard deviation of the wavelengths from the mean, based on measurements of twenty-five flagella, is 0·12 μ and the standard error of the mean wavelength is 0·024 μ. In Fig. 1 is illustrated the flagellar morphology of the organism. The insert in the lower right corner shows the flagellar morphology of a typical strain of *Pseudomonas aeruginosa*.

The wavelength of the flagella of the strain of *Ps. aeruginosa* pictured is most common with monotrichous types of bacteria and averages slightly over 2 μ. This statement is based on the study of over 100 strains, representing many, but mainly unclassified, species. Among the named species of *Pseudomonas* studied may be mentioned *Ps. stutzeri*, *Ps. fluorescens*, *Ps. saccharophila*, *Ps. mucidolens*, and the polar multitrichous *Ps. marginalis*. The flagella were stained according to the method of Leifson (1951).

GROWTH CHARACTERISTICS AND PHYSIOLOGY

The organism grows very readily in a simple peptone solution. The optimum pH is around 7·0 and the optimum temperature around 35°. Liquid media become densely turbid with only a slight pellicle. On the surface of agar media the growth is smooth, raised, and white to creamy in colour. No distinct pigments are produced. Individual colonies are raised, smooth and cream coloured. The original cultures showed two types of colonies: (1) uniformly opaque, and (2) somewhat translucent with an opaque centre and opaque periphery. Isolates from the two types of colonies showed no differences morphologically or physiologically.

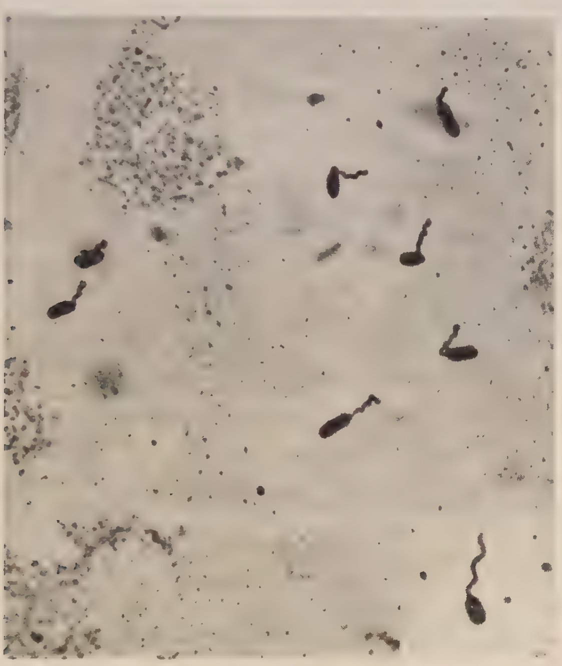

Fig. 1. Typical flagellation of *Ps. diminuta* n.sp. Insert in lower right corner shows the typical flagellation of *Ps. aeruginosa*. Note the curvature of the flagella on the two types of organisms. Leifson flagella stain. Photomicrographs of 2000 magnification.

Detectable acid was not produced from any of the carbohydrates tested including glucose, sucrose, lactose, maltose, arabinose, xylose and galactose. The following tests were negative: indole, MR, VP, urea, H_2S, nitrate and citrate. Litmus milk became blue and translucent. Subsurface colonies in blood agar showed no definite haemolysis. Gelatin was liquefied in about 6 days at 30°. Ethanol was oxidized to acid very promptly and in concentration up to and including 5% (v/v). This latter reaction appears somewhat unusual for an organism which has no effect on carbohydrates. The tests with carbohydrates and ethanol were made using the basal medium described by Hugh & Leifson (1953).

DISCUSSION

On the bases of flagellar arrangement and physiology the organism seems, for the present, best fitted into the genus *Pseudomonas*. The shape of the flagella is so unusual and characteristic that a new species designation seems appropriate. The name *Ps. diminuta* n.sp. is proposed for the organism.

A culture has been deposited in the National Collection of Type Cultures (NCTC 8545).

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(*Received* 16 *June* 1953)

Buxton, E. W. (1954). *J. gen. Microbiol.* **10**, 71–84

Heterocaryosis and Variability in *Fusarium oxysporum* f. *gladioli* (Snyder & Hansen)

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SUMMARY: The basis of variation in morphological appearance of first cultures of *Fusarium oxysporum* f. *gladioli*, isolated from diseased roots and corms of prematurely yellowed Gladiolus plants, was investigated. The occurrence of mutations is considered to be far too infrequent to account for the wide range of variability of the fungus in culture, and the existence of heterocaryons of mixed morphologically distinct genotypes is considered.

Cytological studies show that each microconidium contains one nucleus which is derived by mitosis from the single nucleus of the conidiophore. Hyphal tip cells contain an average of seven nuclei whilst the cells behind, in the older parts of the hyphae, contain only one nucleus each or, more rarely, two. Anastomoses between germ tubes from conidia of different morphological variants occur very frequently and may provide a means whereby heterocaryons are formed.

Synthesis of heterocaryotic cultures is achieved by simultaneous inoculation of plates containing plain water agar medium. Hyphal tips were separately removed from the mixed colonies thus produced and analysed by single-sporing the resulting colonies. Media varying in carbon content and in carbon/nitrogen ratio proved to have a selective action upon one or other of the homotypes constituting a heterokaryon of two known morphologically distinct variants. By use of such media, it was shown that the ratios of nuclei homotypic for 'flat sporodochial' and 'aerial microconidial' variants was altered according to the relative concentrations of carbon or nitrogen in the culture medium.

A discussion follows on the bearing of these results on the reliability of hitherto important taxonomic criteria in a classification of these fungi. The isolates of *F. oxysporum* f. *gladioli* include a wide range of morphological variants which may be included in Snyder & Hansen's *F. oxysporum*. The existence of heterocaryons between taxonomic variants and their plasticity under various cultural conditions, poses the question of their status in any satisfactory classificatory system.

The frequent occurrence of variants in culture of species of Fusarium has long been the cause of serious difficulty in the definition of the taxonomic features of the genus. A realization of this variability led Appel & Wollenweber (1910) to define the cultural states of *Ankultur*, *Normkultur* and *Hochkultur* so that the taxonomic criteria were related to certain stages of the organisms in culture. Snyder & Hansen (1945) suggested that the considerable overlapping of morphological characteristics, hitherto considered to be sufficiently stable for taxonomic purposes, necessitates a complete revision of the genus. Brown (1925, 1926, 1928) and Brown & Horne (1924, 1926) demonstrated the marked effect on fusaria of cultural management and selection. Snyder & Hansen reduced the sixty-five species of Wollenweber & Reinking's classification (1935) to eight. Miller (1946), working with culture variants of Fusarium isolated from wilted muskmelon plants, concluded that the forms in which Fusarium occurs in nature possess few or none of the established features used by Wollenweber as taxonomic criteria. This undifferentiated stage, the 'wild-type' according to Miller, is characterized by 'abundant aerial mycelium on which conidia, mostly non-septate, are borne rather sparsely'. The traditional sporodochia, macroconidia, sclerotia and pionnotes are held to occur in culture only, as a result of mutation. The validity of such taxonomic criteria Miller, therefore, holds open to doubt. This point of view was challenged by Subramanian (1951) who, working with Fusarium isolates of the Martiella, Elegans, Gibbosum and Arthrosporiella sections from various diseased host-plants, concluded that 'species of Fusarium occur in nature in a multiplicity of forms and not as a single wild-type as claimed by Miller. Since sporodochia were produced by a large number of isolates immediately after isolation in first cultures, these are a natural characteristic of the genus and should be of value in taxonomic studies.' None of these authors has considered in detail the basis of the variation reported, but it is fair to say that, following the work of Hansen (1938) on Botrytis, Beadle & Coonradt (1944) on Neurospora and Pontecorvo (1946) on Penicillium and other Fungi Imperfecti, it has been tacitly assumed that much of the variability is due to the heterocaryotic association of unlike nuclei in the same mycelium. The relative importance of mutation in initiating the heterocaryotic condition is not considered, apart from Miller's work.

In a study of a disease of Gladiolus caused by *Fusarium oxysporum* f. *gladioli* (Buxton & Robertson, 1953) considerable variation was met with in the first isolates from diseased corms with respect to colour, colony type and the taxonomically important criterion of macrospore production. At one extreme isolates produced abundant macroconidia on sporodochia and as pionnotes and could be referred to *F. oxysporum* var. *gladioli* as described by Massey (1926), whilst at the other they possessed few, straight, macroconidia in the aerial hyphae and could be referred to *F. orthoceras* var. *gladioli* as described by McCulloch (1944). The basis of this type of variation, which is of common occurrence in Fusarium (Snyder & Hansen, 1940), and which is responsible for much of the difficulty in Fusarium taxonomy has been little investigated. This material seemed favourable for making inquiries along the following lines: (*a*) how far the variation might be due to mutation alone; (*b*) how far it might be due to the segregation of the natural isolates into their component homotypes; (*c*) how stable the heterocaryons were, if formed, and how far they were influenced by the nutritive status of the culture medium; (*d*) what was the phenotypic expression of distinct taxonomic characters in such heterocaryons. An account of these investigations is given below.

MATERIAL

The first isolates of Fusarium from diseased corms of yellowed Gladiolus plants were grown on 2% potato-glucose agar at 25°. Several sectors varying in colour, production of macroconidia as flat pionnotal areas and relative abundance of aerial mycelium were noted and subcultured, being purified later by single spore isolations. Sample isolates of this type are listed in Table 1 below. During the course of subsequent experiments, several hundred subcultures of single spore origin were made of each of these variants and in one case only was there any recognizable occurrence of a mutant from the progeny of these single spore parent colonies. Five pale-violet coloured colonies (not dealt with in this work) occurred as mutants from a set of *c.* 1000 single spores of the purple-vinaceous culture no. 90. The separate variants, therefore, appear to be sufficiently stable for use in the subsequent investigations in which the genetic markers are required to be reasonably distinct and reliable.

INVESTIGATION OF THE HETEROCARYOTIC NATURE OF THE FUNGUS

Distribution of the nuclei in the mycelium and spores

As a preliminary to the experimental synthesis and subsequent analysis of Fusarium heterocaryons it was considered necessary to investigate the distribution of nuclei in the hyphae, conidiophores and conidia of the fungus. Colonies were obtained on glass cover-slips from small drops of plain water agar seeded with mixed-spore suspensions from cultures of red and white variants of the Fusarium. At various stages of growth the colonies were fixed in Carnoy's fluid for 25 min., hydrolysed in N-HCl for 10 min. at 60° and subsequently stained for 30 min. in a mixture of aqueous 2% Azure 'A', $NaHSO_4$ and N-HCl (see Heubschman, 1952). The staining of conidia was facilitated by sticking them to cover-slips with egg albumin. Since an estimate of the distribution and proportions of each nuclear type in the heterocaryon was required, an examination was made of stained preparations showing different stages in conidial formation and its accompanying nuclear behaviour. It was found that the single nucleus of each microconidium was formed by division of the nucleus of the conidiophore. This indicates that each conidiophore is capable of abstricting only one genetic type of conidium. In a heterocaryon this would probably mean that the relative numbers of the two genetic types of conidia produced would bear some relationship to the initial ratio of the two types of nucleus in the original mycelium. Any variations in the ratio of the two types of conidia produced would accrue from a differential rate of nuclear division in the conidiophores containing either of the two types of nucleus. Stages in conidial formation are shown in Fig. 1.

The cells of the mycelium were found to contain, in almost every case, one nucleus only, except in the cases of cell division, these being adjacent to the growing tips of the hyphae, or to cells involved in conidiophore or conidium formation. The hyphal tips possessed no septa for some distance from their growing ends and terminal cells contained a number of nuclei varying, in an even distribution, from 1 to 15. In a sample of fifty-three hyphal tips examined, the mean number of nuclei in the terminal cells was seven. Therefore, on cytological grounds alone it would appear to be impossible for the mycelium to exist as a collection of heterocaryotic cells as it does, for example, in Botrytis (Hansen, 1938), since each portion of the hyphae cut off between septa contains only one nucleus. That is, on the strict definition 'heterocaryon', no such state can exist in the mature mycelium of *F. oxysporum* unless it can be shown that the inter-connexions between the

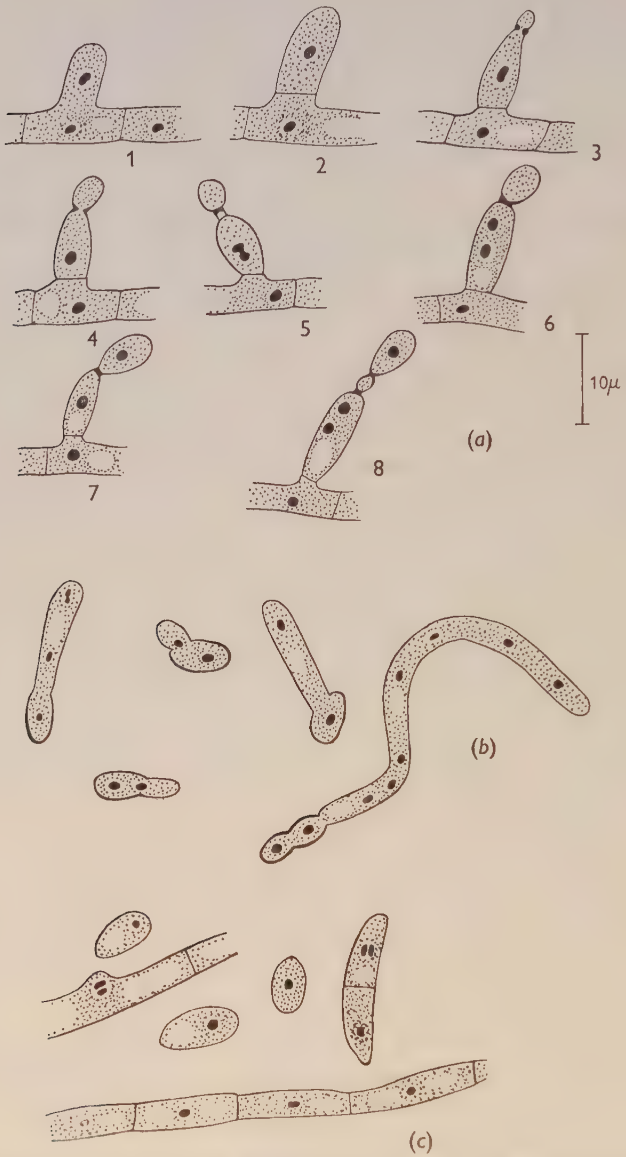

Fig. 1. (*a*) Stages in microconidial differentiation showing the distribution of the nuclei involved—stained by Azure 'A' method (see text). (*b*) Germinating conidia showing a number of nuclei in the germ tubes which are not yet divided by septa. (*c*) Hyphae and conidia showing single nucleus per cell. Nuclei in a final stage of division appear in two of the cells.

'cells' of hyphae are of such magnitude that the mycelium is for practical purposes coenocytic. Whether or not the mycelium acts as a functional heterocaryon is considered later. What is clear is that the microconidia and the macroconidia must obtain nuclei which are effectively segregated in one cell and therefore it is at least theoretically impossible for them to contain nuclei of more than one type, unless mutation has occurred.

Evidence of anastomosis. Stained preparations of germinating conidia showed that a considerable number of anastomoses occurred soon after the germ tubes were produced. Such anastomoses appeared to be more frequent when conidia were germinated in water on a glass surface. The two cultures used had been separately isolated from the roots and vascular core of corms of yellowed Gladiolus taken from the field; one culture exhibited a persistent whitish buff colour, the other a deep purple-vinaceous ground colour which diffused into the agar medium. Mixed spore suspensions from these two different morphological types of *F. oxysporum* f. *gladioli* (i.e. isolates 81A and 90—see Table 2) were sown and anastomoses between the germ tubes were observed (see Pl. 1, fig. 2). Almost all these microconidia, which were indistinguishable morphologically, formed anastomoses; hence it could be assumed that the two variant types of germ tube were involved in anastomoses with each other. Macroconidia from variant 81A produced germ tubes which freely anastomosed with those produced by microconidia of variant 90. The use of macroconidia and microconidia respectively in this latter case determined the exact origin of each of the component hyphae of the observed anastomoses. A considerable number of anastomoses between older hyphae of mixed inoculum were frequently observed in cultures grown on water agar (see Pl. 1, fig. 3). Conidia stained (Azure 'A' method) to show the nuclei are shown in Pl. 1, fig. 1.

Evidence of heterocaryosis. In the course of work already carried out on the Fusarium Yellows disease of Gladiolus (Buxton & Robertson, 1953) by far the most frequent variants found in the original isolates were those concerning colour of mycelium and presence or absence of high abundant aerial mycelium. Sample variants of these types are shown in Table 1. All cultures

Table 1. *Typical variants occurring as sectors from isolates of* Fusarium oxysporum *f.* gladioli

Cultural features are all as shown on potato glucose agar medium. Different patterns were, however, obtained on several other media.

Isolate no.	Colour on potato glucose agar medium	Mycelial type	Sporodochia	Pionnotes (slime-spore masses)
90	Red	Sparse, slightly raised in close mat	Absent	Absent
81A	White-buff	Abundant, high, cottony	Abundant (on oatmeal agar). Pale buff colour	Absent
81F	White	Adpressed, twisted together. No aerial mycelium	Absent	Abundant in light
13	Grey-white	Adpressed, twisted into thick 'ropes'. No aerial mycelium	Absent	Absent

were defined on a standard medium made by adding 20 g. glucose and 20 g. agar to 1 l. of potato extract (200 g.potato/l.).

Single-spore cultures were obtained of each cultural type, and from these in turn single spores were taken in order to ensure genetic purity. Spore suspensions from two of the single-spore colonies of different types were sown mixed on water agar plates and the resulting colony allowed to grow to *c.* 2·5 cm. diam. At this stage, with the use of a finely pointed needle, single hyphal tips of *c.* 150 μ. length were carefully cut away from the advancing edge of the colony and transferred singly to separate tubes of the standard potato-glucose agar medium. These single hyphal tips were grown until a stage of sporulation was reached, i.e. 3–4 days, after which time spore suspensions from each were separately spread over Petri plates containing the standard medium. A suitable dilution produced colonies which could be easily counted and classified into one or other of the two homotypes used for the original synthesis of the heterocaryon. The results of an experiment of this type are shown in Table 2.

Table 2. *Analysis of heterocaryons produced by mixed-spore inoculum of two morphological types of* Fusarium oxysporum *f.* gladioli

		Homotypes resulting from single-sporing the hyphal-tip colonies	
Inoculum	Appearance of colonies from hyphal tips	90	13
Mixed 90 and 13	Greyish purple, slightly aerial	54	*c.* 200
		44	*c.* 500
		48	*c.* 250
		63	*c.* 350
		56	*c.* 300
		47	*c.* 300
Controls 90 and 13	Purple-vinaceous, aerial	*c.* 800	Nil
	Grey-white, not aerial	Nil	*c.* 800

A similar experiment using variants 81 A and 81 F showed again that hyphal tips from mixed-spore colonies segregated into their original components. The smaller numbers involved are due to the fact that single-spore colonies were picked off immediately on germination with the use of the eye of a sewing needle, thereby ensuring that only one spore colony was removed at a time. The results are given in Table 3.

The production of some homocaryotic hyphal tips indicated, as might be expected, that localized breakdown of the heterocaryon had occurred. In order to investigate this possibility, separate hyphal branches at the leading edge of colonies of mixed-spore origin grown on water agar were cut free and cultured separately on potato glucose agar. Examples of this type are shown in Fig. 2.

The examples depicted in Fig. 2 illustrate the mechanism by which sectoring is likely to occur owing to the branching of a heterocaryon into its separate components, thus leading ultimately, provided that continued growth of the separate component takes place, to the production of sectors. These sectors

Table 3. *Analysis of heterocaryons produced from mixed-spore inoculum of two morphological variants, 81 F and 81 A, of* Fusarium oxysporum *f.* gladioli

Inoculum	Appearance of colonies from hyphal tips	Homotypes resulting from single-sporing the hyphal-tip colonies	
		81 F	81 A
Mixed 81 F and 81 A	As 81 A	0	30
	Intermediate, flat with aerial wisps	8	22
	Intermediate, flat with aerial wisps	12	18
	As 81 A	0	30
	As 81 F	30	0
Controls 81 F	As 81 F	30	0
81 A	As 81 A	0	30

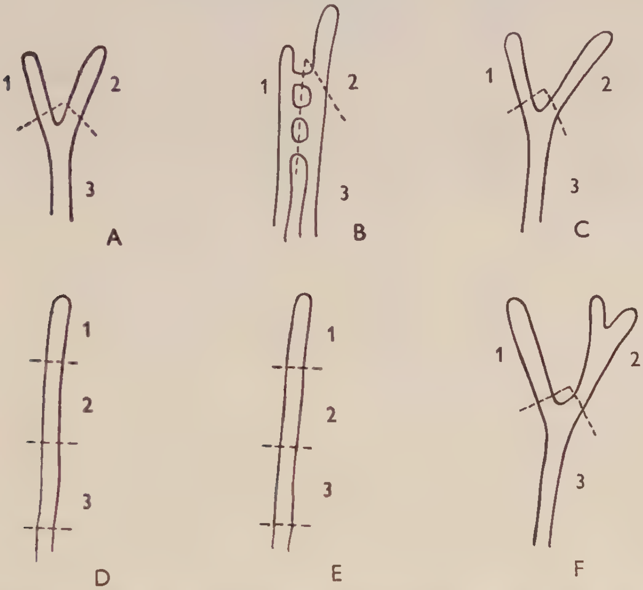

Fig. 2. Examples of branching heterocaryotic hyphae and the resulting colonies produced when the separate components were transferred to potato glucose agar medium. A, B, C and D are heterocaryons of 90 and 81 A. E and F are heterocaryons of 81 F and 81 A. Incisions were made along the dotted lines. Colonies from the excised fragments were as follows: A1: 90 and 81 A; A2: 81 A; A3: 90. B1: 81 A; B2: 90; B3: no growth. C1: 90; C2: 81 A; C3: no growth. D1: 81 A; D2: 90 and 81 A; D3: 90 and 81 A. E1: 81 A and 81 F; E2: 81 A; E3: 81 A and 81 F. F1: 81 A; F2: 81 A; F3: 81 A and 81 F.

are usually evident as fans of a different morphological appearance; they may be overgrown by the heterocaryon or by the sectors of the other homotype. Thus the heterocaryon may be re-synthesized at any time during the growth of the colony by means of anastomoses between the two homotypes (see Pl. 1, fig. 3). With continued growth, the hyphal tip leaves behind a chain of cells of different nuclear type. It seemed possible, therefore, that one or other of the nuclear types might be differentially selected so that the cells laid down, as the colony expands, will differ in proportionate numbers.

If we consider a hyphal tip containing two types of nuclei, it is clear that, with subsequent divisions of each of these two types, such a tip will become homocaryotic for the terminal nucleus (see Fig. 3). Thus the older colonies will show a higher proportion of homocaryotic hyphae on their advancing margin, although there are two possibilities which may be considered which could reconstitute the heterocaryon in these hyphal tips. First, as shown in Fig. 3, the nuclei may pass each other during the course of mitosis—staining shows that this is physically possible. Secondly, frequent anastomoses, as already demonstrated between advancing young hyphae at the colony edges, may facilitate transfer of nuclei, thus reconstituting the heterokaryon.

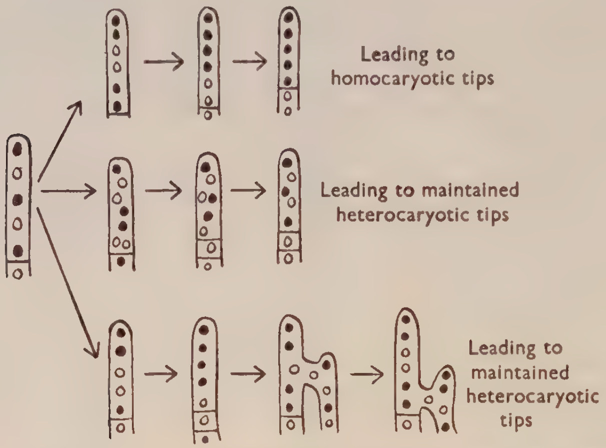
Leading to homocaryotic tips
Leading to maintained heterocaryotic tips
Leading to maintained heterocaryotic tips

Fig. 3. Possible fate of young hyphal tips, leading to a homocaryotic state or, by two possible methods, to a maintained heterocaryotic state

The re-synthesis of heterocaryotic patches, which may or may not break down at a later stage by branching, as depicted in Fig. 2, leads to the formation of a complex nuclear arrangement which is probably best described as a 'mosaic' of the two variants involved. Since this mosaic is constituted at an early stage by numerous anastomoses between germ tubes of the conidia from the two variants, and since branching into the two homotypes may occur at any subsequent stage, it was deemed necessary to compare the nuclear ratios of pieces of hyphae taken from the centre of mixed culture with those at later stages of growth, i.e. at different points along the radius of a colony. The results of such an experiment are given in Table 4, which further substantiates the conception of the two nuclear types as co-existing as a mosaic colony, since some hyphal fragments return a completely homocaryotic range when single-spored.

A similar experiment with variants 81 A and 90 gave the same pattern of random juxtaposition of homocaryotic and heterocaryotic hyphae within the mixed-spore colony.

Selection by the culture medium. The possibility of the variation of the nuclear ratio by a change in the nutritive status of the medium was investi-

Table 4. *A comparison of nuclear proportions in hyphal fragments taken from points at different distances from the centre of a mixed-spore colony of variants* 81 *F and* 81 *A*

Inoculum	Distance from centre of colony at which hyphal fragment cut (cm.)	Appearance of hyphal fragment colonies	Colony types resulting from single-sporing the hyphal fragment colonies				
			Homocaryotic		Heterocaryotic		
			F	A	F	A	Ratio F/A
Mixed 81 F and 81 A	0·2	Intermediate between flat and aerial types	—	—	1037	301	3·4
	0·4	Intermediate between flat and aerial types	—	—	849	63	13·5
	0·4	Flat type	450	0	—	—	—
	0·6	Flat type	900	0	—	—	—
	0·8	Aerial type	0	400	—	—	—
		Flat type	200	0	—	—	—
	1·0	Aerial type	0	400	—	—	—
		Aerial type	0	400	—	—	—
	1·2	Intermediate, but tending towards aerial type	—	—	186	250	0·88

gated by varying the initial ratio in the mixed-spore inoculum using one type of medium only, and by inoculating a range of media of varying carbon contents using one particular mixed-spore ratio. Variants 81 F and 81 A were used in both experiments. In the first experiment, the ratio of the two types of spores was varied between 3 : 1 and 1 : 3, the total numbers in each inoculum droplet being *c.* 150 in each case. The medium used was prepared from an extract of Gladiolus corms with the addition of 20 g. agar/l. The resulting colonies, which were grown in quadruplicate, were single-spored after 4 and 8 days of growth. This was done by removing the whole colonies and thoroughly shaking them separately in tubes of sterile water, thus ensuring a thorough mixing of the two spore types. Suitably diluted spore suspensions from each tube were plated in quadruplicate at a concentration of *c.* 300 spores/plate of medium constituted as follows (g./l. water): glucose, 2·4; peptone, 19; $MgSO_4.7H_2O$, 0·5; K_2HPO_4, 1·0; $FeCl_3$, trace; agar, 20. This medium was used since it gave maximum differentiation between the two phenotypes flat (81 F) and aerial (81 A). The ratios of spore types recovered in this experiment are shown in Table 5.

From this experiment it appears that the Gladiolus corm extract agar medium had no selective effect upon the two nuclear types at least up to 4 days after inoculation. After 8 days, however, visible sectors of 81 A had just appeared and a high return of spores of this type was encountered. In the second experiment of this kind, a mixed-spore inoculum of variants 81 F and 81 A of approximately 1 : 1 ratio was used. The media used varied proportionately in their carbon contents (glucose source) as follows: C0/N2, 0 g.

glucose; C1/N2, 2·4 g. glucose; C2/N2, 4·8 g. glucose; C3/N2, 7·2 g. glucose; in other respects they were as in the formula on p. 79. From the mixed-spore colonies single spores were isolated as before, after 4 days growth, and spread over plates of the C1/N2 medium. The results are shown in Table 6.

Table 5. *Recovered ratios of variants* 81 *F and* 81 *A from mixed-spore inoculum on Gladiolus corm extract agar medium*

Ratios in mixed-spore inoculum. (Haemocytometer counts)		Recovered spore types—on C1/N2 medium					
		After 4 days			After 8 days		
		Total colonies		Ratio F/A	Total colonies		Ratio F/A
F	A	F	A		F	A	
3	1	943	304	3·10	636	230	2·76
2	1	720	402	1·79	1322	325	4·06
1	1	575	550	1·00	747	345	2·10
1	2	849	1369	0·62	1124	497	2·26
1	3	374	1215	0·31	1041	532	2·00

Table 6. *Recovered ratios from* 1 : 1 *mixed-spore inoculum of variants* 81 *F and* 81 *A on a range of four media varying in carbon content*

		Resulting single-spore colonies after transfer to C1/N2 medium		
		Total colonies		Ratio F/A
Medium	Appearance of mixed-spore colony, after 4 days' growth	F	A	
C0/N2	White with sparse, high cottony aerial mycelium	385	230	1·67
C1/N2	Whitish buff with woolly aerial mycelium at centre	475	594	0·80
C2/N2	Whitish buff with woolly aerial mycelium at centre but lower	119	512	0·23
C3/N2	As C2/N2, but aerial mycelium only faintly visible	76	590	0·13

Control plates inoculated with single-spore colonies of the original variants returned 1164 all 81F type and 969 all 81A type, respectively. Hyphal tips taken from the edges of the initial mixed-spore colonies used in the above two experiments showed, after single-spore isolation by the usual technique, that some heterocaryosis had occurred, although a number of homocaryotic tips were also encountered. It is not, therefore, technically possible to exclude the possibility of homocaryotic hyphae (which may occur *de novo* or by breakdown of a heterocaryon) growing side by side with heterocaryotic hyphae. The composition of the mosaic does, however, appear to vary in response to the nutritive environment, probably by the selection of one nuclear type at the expense of the other, as in the case of the experiment in which a high glucose content favoured production of spores of the 81A type.

A further experiment investigating the evidence for the differential selection of the two homotypes by glucose content was carried out. Hyphal tips from heterocaryons of 81 F and 81 A type synthesized on water agar were transferred separately to each of the four media of different glucose content and allowed to grow until colonies of *c.* 1·5 cm. were produced. Ten hyphal-tip colonies were raised on each of the four media. Each colony was single-spored by spreading over plates of the C1/N2 medium as previously described. Subsequent spore counts revealed the results shown in Table 7.

Table 7. *Recovered ratios from* 1 : 1 *mixed-spore inoculum of* 81 *F and* 81 *A types grown on plain agar medium, later transferred as hyphal tips to a range of four carbon media*

Medium to which hyphal tips were transferred	Appearance of heterocaryons	Resulting single-spore colonies on C1/N2 medium		
		Total colonies		Ratio
		F	A	F/A
C0/N2	Fairly flat, white. Sparse cottony aerial mycelium	343	479	0·71
C1/N2	As C0/N2, but slightly more aerial mycelium	158	499	0·33
C2/N2	As C1/N2	21	182	0·11
C3/N2	As C2/N2, but pale buff	132	464	0·28

The heterocaryons showed similar appearances on all four media, being somewhat intermediate between the two component homotypes. It is evident that a heterocaryon which exhibits such a stable morphological appearance on a range of media would, on single-sporing or breakdown by sectoring, yield varying proportions of colonies showing different morphological characteristics.

A further experiment designed to show the effects of two media differing in their glucose/nitrogen ratio upon the relative proportions of the two homotypes in a synthesized heterocaryon was carried out. With 0·2 % carbon (glucose source), 0·1 % nitrogen (peptone source) and 2 % carbon, 0·01 % nitrogen containing media, the same technique was used as in the previous experiment. The results are given in Table 8.

Table 8. *Recovered ratios from* 1 : 1 *mixed-spore inoculum of* 81 *F and* 81 *A variants grown on plain agar medium, the hyphal tips later transferred to media differing in carbon/nitrogen ratio*

An analysis of variance showed an '*F*' value of 16·74, giving significance at 0·1 % level.
A '*t*' test showed that $t_{[19]}=3{\cdot}96$, giving significance at 0·1 % level.

Medium to which hyphal tips transferred		Resulting single-spore colonies from the hyphal-tip colonies											
Low C/N ratio	F	21	11	16	88	91	85	193	137	174	180	149	191
	A	10	3	5	87	102	84	125	88	123	143	109	144
	Ratio F/A	2·10	3·67	3·40	1·01	0·89	1·01	1·54	1·56	1·42	1·26	1·37	1·33
High C/N ratio	F	43	72	73	74	31	71	47	55	53	—	—	—
	A	117	143	104	181	87	134	106	96	86	—	—	—
	Ratio F/A	0·37	0·50	0·70	0·41	0·36	0·53	0·44	0·57	0·62	—	—	—

The results of these experiments indicate that some advantage is conferred upon the 81A component of the heterocaryon used by increasing the glucose content or the glucose/nitrogen ratio of the medium. A count of nuclear ratios, as judged by the proportionate numbers of colonies produced by the single-spore method, gives a clear relationship between the increase of 81A and increasing glucose content. In these experiments 81F produced masses of macroconidia as pink pseudo-pionnotes and possessed no aerial mycelium, whilst 81A formed a woolly colony of buff ground colour and remained non-pionnotal. The production of macroconidia as pionnotes and the colour and texture of aerial mycelium are important criteria in Fusarium taxonomy.

DISCUSSION

Heterocaryosis may be considered as a basis for the explanation of much of the variability in *F. oxysporum* cultures obtained from so-called 'wild-type' isolates. The initial isolates may exist in the host-plant tissue or in the soil as mycelia of different nuclear types which, when segregated under cultural conditions, yield several colonies showing wide differences in morphological characteristics. It is clear that the media employed in the culture of such natural isolates provide a selective environment for the phenotypic expression of the different potentialities of the wild-types. In the section Elegans of the fusaria, some of these variants have been given taxonomic status by Wollenweber & Reinking (1935). The evidence presented here supports the decision of Snyder & Hansen (1940) in placing the old species of this section into the single macro-species *F. oxysporum*, having several formae accommodating the different pathogenic isolates. Heterocaryons between taxonomic types of the isolates from diseased gladioli fall into Snyder & Hansen's *F. oxysporum* and the instability *in vitro* of such heterocaryons poses the problem of defining their taxonomic limits. Which of the many morphological variants capable of causing the disease is to be considered as the basic type for identification by making use of the traditional criteria for taxonomic division of the fusaria? Nuclei genetically pure for pionnotal and for non-pionnotal characteristics, for flat, white or for high, aerial, purple appearance can co-exist as heterocaryons. Such intermediates between taxa, that arise by anastomosis of germ tubes and subsequent migration of nuclei would, on cultivation from single spores, present the potential morphological range of the isolate to the observer. The selective nature of the medium will determine this range and the relative proportions of the taxa.

Jinks (1952) has analysed heterocaryosis in *Penicillium cyclopium* and concluded that the variation in the ratios of the different nuclei in the heterocaryon is adaptive in its significance. Although, as in the case of *Fusarium oxysporum*, the results of Jinks indicate that the relative numbers of the nuclear types are changed in relation to the medium, the possibility of ephemeral sectoring, which may or may not give rise to non-sporing sections of the colony, merits consideration. It is not, therefore, possible to be quite certain whether or not we are dealing with selection of nuclei or of short homocaryotic sporulating side-branches in an analysis of heterocaryons by the single-spore method. The selection of nuclei would be directly related to the ratio of the different types of sporulating side-branches when these are initially formed. In these respects, both Penicillium and Fusarium have their limitations in any interpretation of investigations of this kind.

It has become increasingly evident that comparisons of Fusarium isolates for purposes of identification must be made only on standard media and under identical cultural conditions. Gordon (1952) discussed the difficulties involved in the successful identification of *Fusarium* spp. and concluded that a knowledge of the wild-types from several different soil localities is required before a workable system of classification can be evolved. An investigation of the genetic constitutions of the wild-types in each section of Fusarium would provide a basis for such a classificatory system. *F. oxysporum* may exist in the soil as a heterocaryon or as a potential heterocaryon in the form of local concentrations of spores of different genotypes. As Baker (1944) pointed out, in relation to *Penicillium notatum*, the possession of uninucleate conidia does not preclude the possibility of the occurrence of heterocaryosis. As in the case of the evidence presented above, anastomosis of germ tubes from conidia reconstitutes the heterocaryon. The presence of a root or other suitable part of a host-plant may provide a selective environment for any or all of the nuclear types present. The occurrence of non-pathogenic 'mutants' from the wild-type isolates has long been known to occur. It is suggested that at least some such cases may be explained as arising by segregation *in vitro* of these 'extra' nuclei incidentally occurring in the hyphae invading the host-plant. The proportions of pathogenic and non-pathogenic nuclei in wild-type isolates require investigation, and invasion of host-plant tissue might be studied from the standpoint of selective pressure on the nuclear types in the fusaria.

The author wishes to express his thanks to the Agricultural Research Council for the receipt of a Research Studentship, during part of the tenure of which this work was carried out, to Dr N. F. Robertson for many helpful suggestions, and to Mr F. T. N. Elborn for taking the photographs.

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EXPLANATION OF PLATE

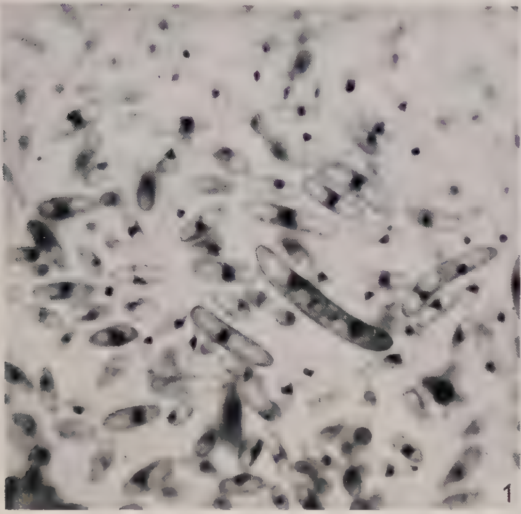
Fig. 1. Spores of *F. oxysporum* f. *gladioli* variants 90 and 81 A showing nuclei (one per cell) stained with 2% aqueous Azure 'A'. × 1500.

Fig. 2. Germinating conidia showing nuclear distribution and anastomoses between germ tubes. × 1500.

Fig. 3. Anastomoses between hyphae of different homotypes—90 and 81 A. × 1500.

(*Received* 16 *June* 1953)

Journal of General Microbiology, Vol. 10, *No.* 1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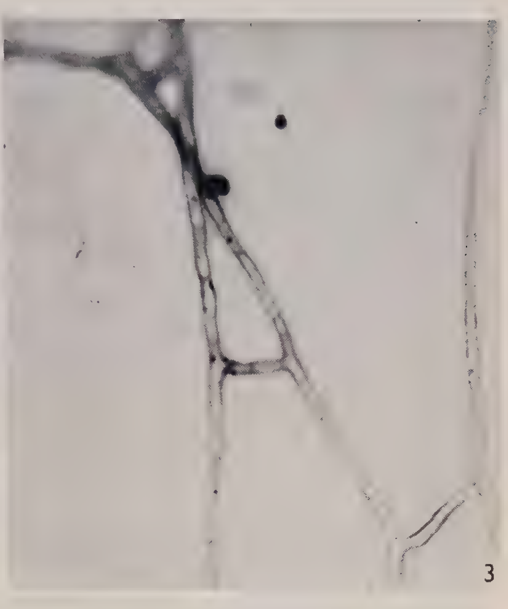
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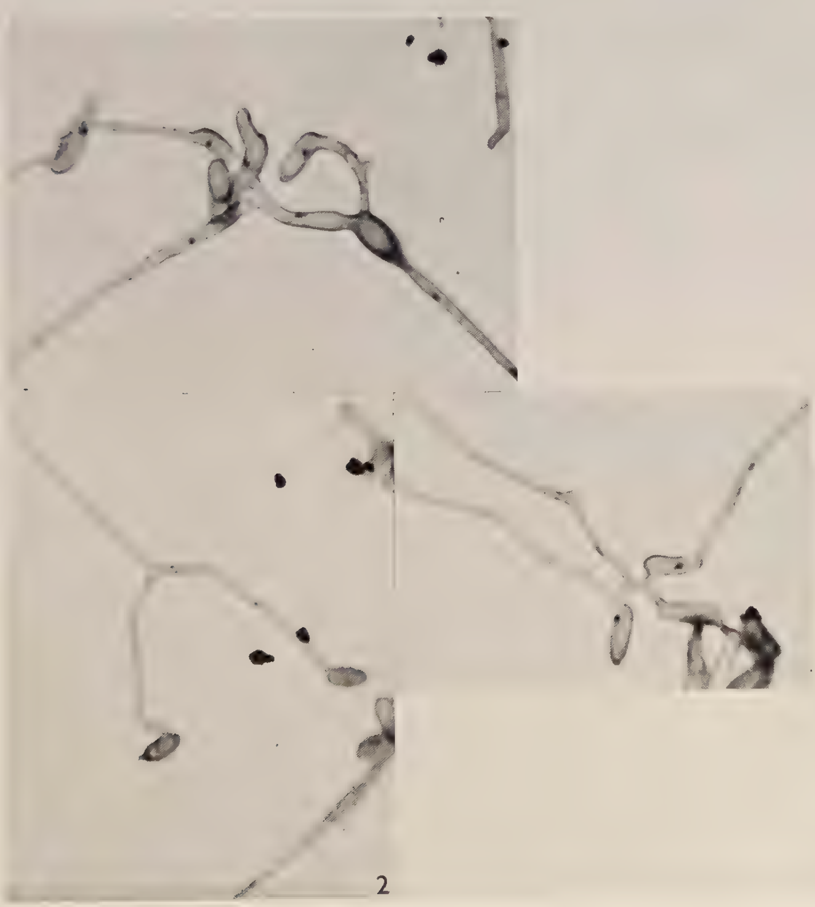
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E. W. Buxton—Variability in *Fusarium oxysporum*. Plate 1

Saperstein, S., Starr, M. P. & Filfus, J. A. (1954). *J. gen. Microbiol.* **10**, 85–92.

Alterations in Carotenoid Synthesis accompanying Mutation in *Corynebacterium michiganense*

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SUMMARY: Colour mutants of the tomato pathogen, *Corynebacterium michiganense*, provided an opportunity to determine the nature of this micro-organism's pigments, and the effect of mutation and nutrition thereon. The major pigments of *C. michiganense* are carotenoids. The parent and mutant strains show distinct qualitative differences in pigment synthesis. The naturally occurring yellow parent type produces cryptoxanthin and lycopene. A pink mutant forms lycopene and spirilloxanthin. A red back-mutant produces only lycopene. An orange mutant synthesizes cryptoxanthin, β-carotene and canthaxanthin. Carotenoids were not detected in the colourless mutants.

Carotenoid synthesis in the yellow strain of *C. michiganense* is affected by thiamine in a manner similar to that previously described for *C. poinsettiae*: cryptoxanthin and lycopene are synthesized only at relatively high thiamine concentrations. The formation of lycopene, relative to spirilloxanthin, in the pink mutant is favoured by high thiamine concentrations. Similarly, the red strain forms appreciably more lycopene at higher thiamine concentrations than at lower. The formation of carotenoids in the orange strain appears not to be influenced by external thiamine concentration, possibly because this culture synthesizes an excess of the vitamin. No other nutritional factor which was examined caused detectable alterations in colour of the cells.

Colour variants of *Corynebacterium michiganense* (E. F. Smith) Jensen were described by the earliest students of this phytopathogenic coryneform organism. Bryan (1930, 1931) isolated 'spontaneously' occurring white and pink strains of this normally yellow organism, and similar colour variants were obtained by Ark (1946, 1951) from broth cultures to which had been added acenaphthene or uranium salts.

Nutrition is known to affect pigmentation of this species. The naturally occurring yellow type of *C. michiganense* was reported by Braun (1949) to produce either a cream or a yellow growth, depending upon whether the concentration of thiamine in the growth medium was low (0·1 μg./100 ml.) or high (1–100 μg./100 ml.), respectively. Braun noted that a white mutant of this organism produced no pigment, regardless of the thiamine concentration. In the related species, *C. poinsettiae* Starr & Pirone, thiamine was observed to affect pigmentation (Starr & Pirone, 1942; Starr, 1949); later a more intensive examination of this effect was made by the present writers (Starr & Saperstein, 1953). At low thiamine concentration (0·1 μg./100 ml.) the cells of *C. poinsettiae* are pink; whereas, at higher concentration (100 μg./100 ml.) the cells are orange-yellow; these colour changes have been related to qualitative and quantitative alterations in carotenoids.

There has been no study, before the one reported here, on the nature of the individual pigments of *C. michiganense*; in fact, Braun (1949) does not mention whether they are carotenoids. Consequently, it is not known whether the pigments in the mutants are related to those of the parent strain. The present investigation was undertaken with the following objectives: (*a*) to characterize the major individual pigments of the naturally occurring yellow type and of several colour variants of *C. michiganense*; (*b*) to determine whether the colour shift accompanying the change in thiamine concentration is related to the situation in *C. poinsettiae*; (*c*) to uncover any other connexion between the pigments and the nutrition of individual strains; (*d*) to provide data bearing upon the mechanism of microbial carotenoid synthesis.

MATERIALS AND METHODS

Cultures. The cultures used throughout this study were authentic strains of *C. michiganense* obtained from Prof. P. A. Ark, University of California, Berkeley. Culture 4702 is a yellow strain isolated from tomato. Cultures 4944, 4938 and 4939—pink, orange, and colourless, respectively—are colour mutants isolated by Prof. Ark following treatment of a parent culture (4702) with uranium sulphate. Culture 4999 is a colourless mutant isolated by the same investigator following treatment of the same parent strain with 5-amino-acenaphthene. Culture 4999RR is a red variant, probably resulting from spontaneous reverse mutation, isolated by the present writers from the colourless culture 4999. All cultures were pathogenic for tomato at the start and conclusion of this investigation; in this connexion, we substantiated the findings of Fawcett & Bryan (1934) who found that pink variants usually are less virulent than either the yellow or the colourless strain.

Medium. For mass cultivation, a medium having the following composition per 100 ml. was used: peptone 1 g.; glucose 1 g.; pH adjusted to 6·8. For studies on the effect of thiamine on carotenoid production, a medium was used which had the following composition per 100 ml.: acid-hydrolysed casein (vitamin-free casamino acids, Difco) 0·5 g.; KH_2PO_4, 0·5 g.; sodium citrate, 0·01 g.; $MgSO_4.7H_2O$, 0·02 g.; 'trace metals solution' (Starr, 1946) 1 ml.; biotin 0·1 μg.; nicotinic acid, 10 μg.; thiamine, 0·2–200 μg.; glucose (added separately after autoclaving) 1 g.; pH 6·8. The procedure for mass cultivation and harvesting of the cells was identical with that described in an earlier report (Starr & Saperstein, 1953).

Nutritional studies. The nutritive requirements of the cultures were determined by conventional means; the technical details were similar to those in previous studies of phytopathogenic micro-organisms (Starr, 1946, 1949). Cultures were incubated at 28° on a shaking machine to increase the cell yield. Estimations of turbidity were made with the Klett-Summerson photoelectric colorimeter using the 660 mμ. filter.

Solvents. The solvents employed were as follows: methanol, light petroleum (35–60°), acetone, benzene (C. P., Baker), chloroform (Reagent, Merck), hexane (Standard mixed hexanes, Standard Oil Company of California) redistilled to yield the 62–65° fraction. Where necessary, solvents were purified by conventional methods.

Extraction and separation of the carotenoids. The cell mass was dried with 7 to 9 vol. of acetone, powdered in a ball mill, and then extracted with a mixture of methanol and light petroleum. The extracted fractions were combined, and the acetone and methanol removed by washing with water in a separating funnel. Saponification was carried out by adding an equal volume of alkaline methanol (18 % (w/v) KOH in 90 % (v/v) methanol) to the light petroleum solution and agitating the mixture under nitrogen by means of a magnetic stirrer for at least 20 hr. at room temperature. After thorough washing, the light petroleum solution containing the mixture of carotenoids was dried over anhydrous sodium sulphate.

RESULTS

Chromatographic separation of the polyenes

The dried light petroleum solutions of the mixture of carotenoids were chromatographed on columns of magnesia + filter aid mixed 2 : 1 by weight (Westvaco Chemical Company, 2641 adsorptive powdered magnesia; Hyflo Super Cel of Johns Manville Company). The chromatograms were developed with 5–8 % (v/v) acetone in light petroleum. Records of typical chromatograms are shown in Table 1. The columns were cut to separate the individual bands and the pigments were eluted with a mixture of chloroform + methanol or

Table 1. *Records of typical chromatograms showing pigments present in* Corynebacterium michiganense *mutants*

Bands on magnesia + filter aid column, listed from top downwards.

Organism 4702 (parent; yellow)

Band		
Width (mm.)	Colour	
10	Yellow-orange	Cryptoxanthin
20	Diffuse band, pink	All-*trans*-lycopene
	Faint yellow eluant	*cis*-Lycopene

Organism 4944 (pink)

Band		
Width (mm.)	Colour	
3	Red	All-*trans*-spirilloxanthin
1	Clear	
10	Pink	*cis*-Spirilloxanthin
2	Clear	
3	Orange	All-*trans*-lycopene
3	Yellow	*cis*-Lycopene

Organism 4938 (orange)

Band		
Width (mm.)	Colour	
2	Orange	Cryptoxanthin
8	Purple	Canthaxanthin
1	Clear	
2	Dark red	*cis*-Canthaxanthin
30	Clear	
2	Orange	β-Carotene

Organism 4999 RR (red)

Band		
Width (mm.)	Colour	
4	Red-orange	All-*trans*-lycopene
1	Clear	
3	Yellow-orange	*cis*-Lycopene

Organisms 4939, 4999 (colourless)
No pigment

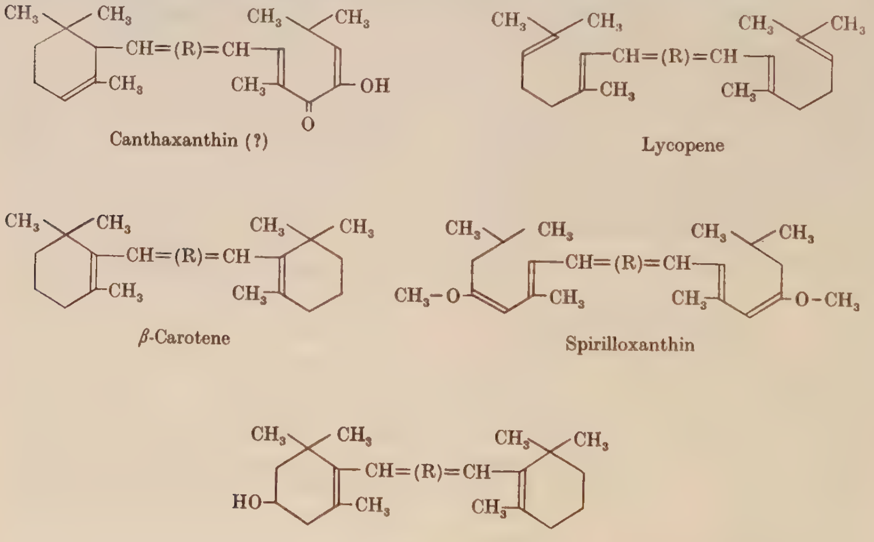
benzene + methanol (1 : 1). The pigment from each band was chromatographed separately to insure chromatographic purity. For this purpose the eluates were evaporated nearly to dryness under reduced pressure, and then redissolved in light petroleum. The light petroleum solutions were percolated through columns of $Ca(OH)_2$ + filter aid (2 : 1 by weight) and the chromatograms developed with 2–8 % (v/v) acetone in light petroleum.

Identification of the pigments

The individual pigments (listed in Table 1 and depicted in Fig. 1) were characterized by their absorption spectra (Table 2), by their position on chromatographic columns relative to known carotenoids, and by their behaviour when partitioned between aqueous methanol and light petroleum. More positive identification was obtained by demonstrating chromatographic homogeneity with known pigments.

From the original yellow culture, 4702, two pigments were detected and isolated: cryptoxanthin and lycopene. These pigments were present only when the concentration (*c.* 200 μg./100 ml.) of thiamine in the medium was well above the concentration necessary for minimal growth. When the concentration of thiamine was diminished to a level which permits only minimal growth (*c.* 0·2 μg./100 ml.), the cells were cream to white in colour and only traces of carotenoid pigments were detected. These results are in accord with Braun's (1949) finding that a shift in colour of the cell mass was mediated by the thiamine concentration.

The pink mutant 4944 produced spirilloxanthin and lycopene, whereas the



Canthaxanthin (?)

Lycopene

β-Carotene

Spirilloxanthin

Cryptoxanthin

Fig. 1. Structural formulae of the principal carotenoids of *Corynebacterium michiganense* (Karrer & Jucker, 1950; Bonner, 1950). The structure of canthaxanthin is not known with certainty (Saperstein & Starr, 1954). 'R' signifies the common $C_{20}H_{24}$ central portion of the molecule.

red back-mutant 4999 RR produced only lycopene. With organism 4944, there was no apparent change in colour at high or low thiamine concentrations. The scanty sediment which developed in the control culture (no added thiamine, but probably containing traces of it) was pink in colour. Since a considerable change in pigment production might have occurred without a concomitant change in the colour of the cell mass, the relative concentrations of spirilloxanthin and lycopene were estimated in the cells of 4944 grown at two concentrations of thiamine (0·1 and 100 μg./100 ml.). A distinct difference was observed: the content of spirilloxanthin relative to that of lycopene was significantly different at the two concentrations of thiamine. The ratios of the optical densities at 530 mμ. (spirilloxanthin) and 442 mμ. (lycopene) in the light petroleum (60–70°) from unchromatographed extracts were as follows:

Table 2. *Spectral characteristics of the carotenoid pigments observed in* Corynebacterium michiganense

	Absorption maxima (black), minima, and inflexions (brackets)*	
Pigment	**In benzene (mμ.)**	**In hexane (mμ.)**
Cryptoxanthin	**493**, 481, **464**, [439]	**477**, 467, **451**, [426], 358
Lycopene		**502·5**, 488, **471**, 454, **445**, 377
Spirilloxanthin	**546**, 530, **510**, 490, **480**, 410, 397	**529**, 514, **495**, 476, **467**, 400, 388, 378
β-Carotene	**492**, 482, **464**, [440], 360	**478**, 468, **451**, [426], 358
Canthaxanthin	**480**	**468**

* Obtained with the Beckman photoelectric spectrophotometer Model DU.

Low-thiamine grown cells: O.D.$_{530}$/O.D.$_{442}$ = 0·72.
High-thiamine grown cells: O.D.$_{530}$/O.D.$_{442}$ = 0·38.

Since it has already been shown that the lycopene concentration in the naturally occurring yellow parent strain undergoes an absolute decrease when the cells are grown at the lower thiamine concentration, it is likely that the alteration in relative concentration of lycopene in the pink mutant 4944 is brought about in the same way.

Organism 4999 RR, which forms only lycopene, showed no visible change in colour when the thiamine concentration was diminished; however, when the pigment content of the cells was estimated by the method of Stahly, Sesler & Brode (1942), we detected an increase in pigment concentration of approximately 36 % at high thiamine concentrations. This strain does not require thiamine as an obligatory nutrient, but is stimulated by its addition to the medium (see below).

The orange mutant 4938 produced three carotenoid pigments: cryptoxanthin, β-carotene and canthaxanthin. Only one of these, cryptoxanthin, was found in the original yellow strain. Canthaxanthin, previously isolated from the fungus *Cantharellus cinnabarinus* by Haxo (1950), has not before been reported from bacteria. The characteristics of this ketonic pigment are the subject of a separate detailed report (Saperstein & Starr, 1954). With the orange mutant there was no change in pigment production when the quantity of thiamine added to the medium was varied; it should be mentioned that strain 4938 synthesizes thiamine (see below).

The colourless mutants 4939 and 4999 showed no pigment production during this study at any concentration of thiamine in the growth medium (Table 1).

No fluorescent zones were found when any of the chromatographic columns were examined, at various stages of development, with ultraviolet radiation at a distance of approximately 3 cm. (Hanovia utility ultraviolet lamp, blue filter). The percolates showed no characteristic absorption curves to suggest the presence of colourless polyene pigments.

Nutritional requirements

To determine whether there was a change in nutritional patterns of the mutants as compared with the naturally occurring yellow strain, and to see whether such changes might reflect any alteration in carotenogenesis, all the organisms were screened for vitamin and amino acid requirements. Other cultures of the naturally occurring yellow type and a white variant had been shown previously to require biotin, thiamine and nicotinic acid (or nicotinamide) for minimal growth in a casein hydrolysate medium (Starr, 1949).

The colourless strains 4939 and 4999, and the yellow strain 4702, showed the same vitamin requirements (biotin, thiamine, and nicotinic acid) as the white and yellow cultures studied by Starr (1949). The orange mutant, 4938, did not require any accessory growth factors for minimal growth, and did in fact excrete biotin, thiamine and nicotinic acid (or nicotamide) into the growth medium, as shown by microbiological assay. Strain 4944 required only biotin and thiamine for minimal growth, and strain 4999RR required only riboflavin obligatorily for minimal growth, but growth was increased considerably upon the addition of nicotinic acid, biotin and thiamine.

In the presence of the required vitamins, all the cultures used in the present study were able to utilize monosodium glutamate (1 g./l.) as the sole source of nitrogen; indeed, glutamate was found to be essential for growth in chemically defined media. Ammonium chloride alone, or in a combination with mixtures of amino-acids lacking glutamate, did not support growth of any of the *C. michiganense* cultures. Methionine can replace glutamate in strain 4702.

Lowering the concentration of the required growth factors other than thiamine did not result in any change in colour of the cell mass, although the growth was markedly diminished. Addition to the medium of L-leucine, an amino-acid shown by Goodwin & Lijinsky (1951) to increase carotenoid production in *Phycomyces blakesleeanus*, did not affect pigmentation in *Corynebacterium michiganense* strains, nor in *C. poinsettiae*, when pigment concentration was estimated by the method of Stahly *et al.* (1942).

DISCUSSION

It is quite clear from the foregoing that *C. michiganense* contains carotenoid pigments, and that the differences in colour between the naturally occurring yellow culture and the mutants reflect qualitative changes in carotenoid production. The qualitative manifestation of pigment production in *C. michiganense* could not, however, be correlated with any change in amino acid or vitamin requirements, other than the thiamine effect first demonstrated for this species by Braun (1949).

One interesting aspect of our results is the demonstration that the shift in pigmentation from cream to yellow in the parent strain 4702 which is mediated by thiamine, was due to the formation of cryptoxanthin under conditions of high thiamine concentration in the medium. In this respect, the organism behaved like *C. poinsettiae* (Starr & Saperstein, 1953) where the formation of cryptoxanthin was shown to be influenced by the concentration of thiamine in the culture medium. Furthermore, *C. poinsettiae* showed a slight decrease in lycoxanthin (3-hydroxylycopene) production when grown in a low thiamine medium. Formation of the related carotenoid lycopene by the naturally occurring yellow strain of *C. michiganense*, as well as by the colour mutants 4944 and 4999RR, was diminished by low thiamine concentrations sufficient only to support minimal growth. These parallels suggest the operation of similar mechanisms for the production of these pigments by the two species.

Of interest, too, is the demonstration that the orange mutant of *C. michiganense* produces β-carotene, cryptoxanthin and canthaxanthin, while the pink mutant produces only lycopene and spirilloxanthin, and the red back-mutant, lycopene alone. This may indicate an independent genetic control for the production of carotenoids with cyclohexenyl rings, and carotenoids with non-cyclic structures, respectively. The absence of colourless polyenes from the coryneform organisms we have studied does not support the mechanism for carotenogenesis proposed by Porter & Lincoln (1950), by Turian (1950) and by Bonner *et al.* (1946), which assumes that the more saturated polyenes are precursors of the less saturated carotenoids, which result from the step-wise removal of hydrogen atoms from the former. The differences which exist between the individual carotenoids of the parent and mutant strains suggest that alterations in polyene structure probably occurs prior to the formation of the completed C_{40} chain. This hypothesis has been further developed elsewhere (Saperstein, 1953).

We wish to thank Prof. P. A. Ark, of the University of California at Berkeley, for the cultures of *Corynebacterium michiganense*, and Miss Barbara Brack, of our laboratory, for performing the microbiological assays.

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(*Received* 29 *June* 1953)

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A Marine Stichococcus sp. which requires Vitamin B_{12} (Cobalamin)

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SUMMARY: A marine *Stichococcus* sp. was found to require a growth factor present in small quantities in natural sea water. Probably the same factor was produced in the medium by a bacterium with which the alga was associated when isolated. The factor could be removed from sea water by activated charcoal, and could be replaced by crystalline vitamin B_{12} (cobalamin).

The marine alga used in this investigation was collected in Great South Bay, Long Island, N.Y., by Mr J. H. Ryther (1954) and was purified as described below. There is some doubt as to the identity of the alga, which is a unicellular form about 2 μ. wide and 2–6 μ. long. It has been tentatively assigned by the present author to the genus *Stichococcus*, and may be identical with *S. cylindricus* Butcher (1952).

MATERIALS AND METHODS

Glassware. Pyrex test tubes, 15 cm., acid-cleaned, capped with glass vials.

Media. These were dispensed 5 ml./tube.

SW. Native sea water (North-west Arm, Halifax, N.S.; winter, 1952–3).

SWM. SW supplemented with minerals (g./l.): K_2HPO_4, 0·02; $Ca(NO_3)_2 . 4H_2O$, 0·10; traces of Fe, Mn, Mo, B, Cu, Zn (Burkholder & Nickell, 1949).

SSM. Artificial sea water (g./l.): NaCl, 23·0; $MgCl_2$, 5·0; Na_2SO_4, 4·0; $NaHCO_3$, 0·2; supplemented as under SWM.

Sterilization was by autoclaving at 15 lb./sq.in. for 15 min.

Other reagents. Activated charcoal (Norit-A; British Drug Houses Ltd., Toronto); Vitamin B_{12} (crystalline, Merck).

Culture conditions. Constant illumination (220 f.c.) from white fluorescent tubes. Temperature, 23°.

Inoculation. From depleted SWM culture, with platinum loop.

Growth determinations. Optical densities of cell suspensions were made by means of a Klett densitometer, with ruby filter (No. 66). For cell counts a Fisher haemocytometer was used.

EXPERIMENTAL

Isolation of the alga

In the original culture the *Stichococcus* sp. was found to be associated with two other algae (*Chlorella* sp. and *Mischococcus* sp.) from which it was readily separated by streaking on SWM agar. By successive streakings it was possible to eliminate all contaminants save one. The alga was apparently unable to grow well except in close association with bacterial colonies, suggesting that it might derive from the bacteria some organic micronutrient essential for growth. It was finally obtained in bacteria-free culture by streaking on SWM agar supplemented with Bacto-tryptone, and a pure clone (not necessarily identical with the pure culture independently isolated and studied by Ryther) was used in all subsequent tests.

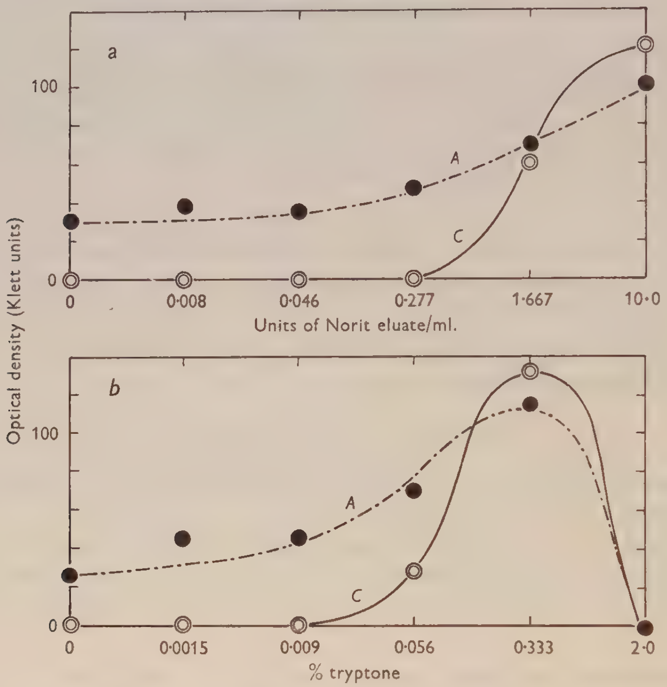

Fig. 1. *a:* Effect of Norit-eluate on final yield of *Stichococcus* sp. in: A, native sea water, supplemented with mineral nutrients (SWM); C, Norit-treated sea water, supplemented as in A. One unit represents the amount of growth factor extracted from 1 ml. of sea water. *b:* Effect of tryptone on final yield of *Stichococcus* sp. Media as in 1*a*.

Identification of the growth factor

It was observed that the alga was capable of slight growth in SWM without further supplement, indicating that the sea water already contained small amounts of growth factor or factors. These traces could be removed by treating the sea water (1 l.) with activated charcoal (10 g.); and medium SWM, prepared with sea water so treated, did not support appreciable growth of the alga. Moreover, the factor could be eluted from the absorbent by ethanol. The charcoal was washed with 95% ethanol (50 ml.), the eluate evaporated to dryness on a water bath, and the residue taken up in medium SSM (10 ml.). The addition either of eluate or of Tryptone to charcoal-treated sea water again permitted growth of the alga (see Fig. 1).

A number of water-soluble vitamins were tested in charcoal-treated sea-water media, but only vitamin B_{12} (cobalamin) proved effective in promoting the growth of the alga. The fact that growth could be obtained in a defined mineral salt mixture, SSM, supplemented by only vitamin B_{12}, indicated that no other growth factor was required (see Fig. 2).

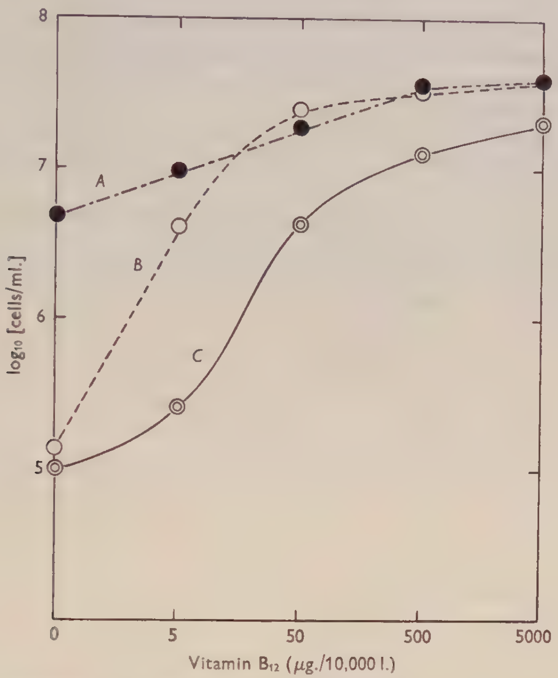

Fig. 2. Effect of vitamin B_{12} on final yield of *Stichococcus* sp. in: A, native sea water, supplemented with mineral nutrients (SWM); B, artificial sea water, supplemented as in A (SSM); C, Norit-treated sea water, supplemented as in A. In B and C, growth in the absence of added vitamin B_{12} is attributable to carry-over of vitamin with the inoculum.

DISCUSSION

For maximum growth *c.* 0·2 μg. cobalamin/l. was required. Since vitamin B_{12} is only partly destroyed by autoclaving (Robbins, Hervey & Stebbins, 1950), the error introduced when media were sterilized by heat does not seriously affect the values given here, which are merely indications of orders of magnitude. One cell apparently requires not less than $1{\cdot}25 \times 10^{-19}$ g. of vitamin B_{12}; or, taking the molecular weight of the vitamin as 1400,

$$\frac{6 \times 10^{23}}{1400} \times 1{\cdot}25 \times 10^{-19} = c.\ 50 \text{ molecules.}$$

A cell of *Euglena gracilis* requires *c.* 4900 molecules of vitamin B_{12} (Hutner, Provasoli, Schatz & Haskins, 1950): a cell of *Lobomonas rostrata c.* 5700 (Lewin, R. A., unpublished).

Assuming that the assay is quantitative, it may be deduced that the free soluble vitamin B_{12} in sea water of the North-west Arm (Halifax, Nova Scotia) was of the order of 0·01 μg./l. in the winter of 1952–3. This may be a higher value than would be found in water from the open sea, since the North-west Arm is a narrow inlet receiving considerable surface run-off from the city of Halifax and its environs.

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(*Received* 1 *July* 1953)

TOMCSIK, J. & GUEX-HOLZER, S. (1954). *J. gen. Microbiol.* **10**, 97–109

Demonstration of the Bacterial Capsule by means of a pH-dependent, Salt-like Combination with Proteins

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SUMMARY: The capsules of bacteria and of *Cryptococcus neoformans* are invisible under the phase-contrast microscope. They can be made visible through addition of a number of proteins at a certain, usually very narrow, pH range lying on the acid side of the isoelectric point of the proteins. The pH range at which the reaction occurs shifts with the isoelectric point of the protein used. The optimal pH range depends, on the other hand, upon the capsular substance of the micro-organism. The non-specific capsular reaction is elicited through a salt-like combination of several proteins with the bacterial capsule, leading to precipitation at a pH value on the alkaline side of the isoelectric point of the bacterial surface and on the acid side of the isoelectric point of the proteins. The reaction is reversible; it disappears on changing the pH. The non-specific capsular reaction at an inter-isoelectric pH zone of the bacterial surface and of the proteins is not a 'swelling' reaction. A swelling of the capsule might occur with some bacteria as a secondary phenomenon on the alkaline side of the reaction zone. The reaction occurs in a broader zone in members of the genus *Bacillus* where it reveals a distinct shrinkage of the capsule at the acid side of the reacting zone.

Nakamura (1923) studied the effect of acids and alkalis on the egg-white lysis of sarcina. He observed that HCl at N/200 to N/1600 prevented the lysozyme activity. When, however, an hour later the suspension was made slightly alkaline with 0·05 N-NaOH, an instantaneous lysis occurred. When studying the effect of lysozyme on the cell structure of capsulated lysozyme-sensitive members of the genus *Bacillus* (Tomcsik & Guex-Holzer, 1952), we repeated the experiment of Nakamura and extended his observation by examining bacteria, after the addition of egg-white at different pH values, under the phase-contrast microscope. During this experiment, we made the unexpected observation that the capsule of these bacteria was beautifully demonstrated in the presence of a 1/50 dilution of egg-white at about N/320-HCl. The fractionation of egg-white revealed that the albumin, globulin and lysozyme fractions all were able to induce a non-specific capsular reaction; however, the optimal pH zone for eliciting this reaction was different with the three different proteins. The purpose of this paper is to describe this phenomenon and to study the behaviour of a representative member of capsulated micro-organisms towards several proteins, including proteins with different isoelectric points.

MATERIALS AND METHODS

Organisms. Bacillus anthracis: strain Vollum (NCTC 7200); an attenuated strain (A_2) isolated by us from a Pasteur vaccine. A strain of *B. megaterium*, which produced a capsule on ordinary agar medium, obtained through the kindness of J. P. Aubert (Service de Fermentations, Institut Pasteur, Paris).

Bacillus M. isolated and described by us (Tomcsik, 1950; Tomcsik & Guex, 1951). A strain of *B. subtilis* which produced a capsule in 3 hr. agar cultures and abundant slime which consisted of D-glutamic acid polypeptide (strain studied by Tomcsik & Guex, 1951). A strain of *Streptococcus haemolyticus*, group C (human), isolated from a case of otitis media (obtained from A. Grumbach, Hygiene Institute, University of Zürich). Strains of *Klebsiella pneumoniae*, types A, B and C (from the Statens Seruminstitut, Copenhagen) and another type B isolated in our Institute from a guinea-pig epidemic. *Diplococcus pneumoniae*, type III, a strain isolated in our laboratory from a human case of pneumonia. *Cryptococcus neoformans*, strains D.U. and R.E. (obtained from Dr E. W. Evans, Department of Bacteriology, University of Michigan, Ann Arbor, U.S.A.).

Culture media. Gladstone & Fildes (1940) CCY agar medium was used throughout the work with the following exceptions: the capsule production of the *B. anthracis* strains was not optimal on CCY medium in presence of 20% (v/v) CO_2; thus Difco peptone agar was used for this purpose. The streptococcus and pneumococcus were grown on blood agar and *Cryptococcus neoformans* on Sabouraud medium. *B. subtilis*, *B. megaterium* and *Bacillus M.* were incubated at 32°, all the other strains at 37°.

Proteins. Albumin and globulin fractions of horse serum as well as those of egg-white were prepared by ammonium sulphate fractionation (Sahyun, 1944). Crystallized lysozyme was prepared according to the method of Alderton & Fevold (1946); this preparation was compared with a sample obtained from the Mann Research Laboratories, New York. The other preparations were as follows: pepsin and trypsin (Kahlbaum); isoelectric casein (Difco); haemoglobin and protaminsulphate (Hoffmann La Roche, Basel); bovine plasma albumin fraction V (Armour Laboratories, London).

Preparation of the reacting mixture of bacteria and proteins at different pH *values.* The bacteria were usually suspensions in 0·85% NaCl from 24 hr. agar cultures. The turbidity was less than in tube no. 3 of the McFarland $BaSO_4$ standard series (Kolmer, 1924), giving ten or more bacteria in the reacting mixture per field in microscope preparations with the oil-immersion objective no. 90 and ocular no. 10. Different dilutions of the proteins in physiological salt solution or in distilled water were added to equal volumes of bacterial suspension; 0·3 ml. of this mixture was added to 0·3 ml. of different buffer solutions which covered a pH range between 2 and 9 at intervals of 0·4 pH unit up to pH 4·4, and at larger intervals on the more alkaline side of the series. The buffer solutions of Teorell & Stenhagen (1938) were used throughout, if not otherwise mentioned. Two loopfuls of these mixtures placed on slides were examined under cover-glasses with the oil-immersion objective of the phase-contrast microscope of Wild and Co. (Heerbrugg, Switzerland). The microscopic examinations could be carried out immediately after setting up the series, but the results were hardly changed when the series was kept at room temperature for 20 hr. The microscopic examinations were usually carried out 1 hr. after setting up the series. The photographs were made with Leitz Ortholux microscope and Aristophot microphotographic apparatus, using the phase-contrast condenser and objectives of Wild and Co.

The same results were obtained by using, instead of buffer solutions, appropriate distilled water dilutions of N-HCl or of N-acetic acid covering the same pH range. An even distribution of pH differences, taking into consideration the buffering actions of proteins, could, however, not be achieved with the latter method.

Typical examples of the buffer series are given in the Tables 1–3. It should be pointed out that at the protein concentrations used in Tables 2 and 3 (0·05 %, w/v) the actual pH value was about 0·4 unit higher than the pH of the buffer solutions, at least in the range pH 2–4. Electrometric determinations were carried out only in certain selected series. The reacting mixtures were prepared for this purpose in the same proportions as above, using, however, 20 times larger volumes of each of the reagents. The electrometric pH determinations are summarized in the Table 4.

RESULTS

Strains of eight species of capsulated micro-organisms, including three serological types of *Klebsiella*, were studied as regards their behaviour towards twelve proteins at different pH values. The proteins had to be studied at different concentrations; thus it was necessary to carry out the microscopical examination of several hundred series. In consequence only the essential results, illustrating certain principles, can be described.

Non-specific capsular reaction of different micro-organisms

The capsulated micro-organisms studied can be divided on the basis of the chemical nature of their capsule into three groups; organisms of the first group produce polysaccharide; those of the second group a polypeptide capsule; the capsule of the third group is of complex nature containing both polysaccharides and polypeptides. The pneumococci, the klebsiellas and the streptococci belong to the first group, with capsules of generally known chemical compositions. The capsules of these micro-organisms are classified by Stacey (1946) as mucopolysaccharides, and the majority of them contain uronic acids.

Table 1 shows the non-specific capsular reaction with bovine serum diluted 1/20. The pH value of the reaction mixture containing, for instance, buffer solution of pH 2·8, corresponds to pH 4 owing to the strong buffering action of the serum at this concentration. The capsular reaction of the klebsiellas (Pl. 2, fig. 17) and the streptococci occurred at pH 4 and 4·4 respectively, extending only with the klebsiella types A and C, up to 4·8. Both klebsiella type B strains showed a definitely lower optimum. The same observation was made with horse and human sera and with a number of other proteins which reacted with the capsule. Other strains must be studied in order to establish whether this difference can really be correlated with the chemical nature of the type B klebsiella polysaccharide. The size of the capsule was not larger at the optimal reaction than that found by the indian ink method. Only in preparations kept in a wet chamber for several hours could a secondary swelling occasionally be observed. The only pneumococcus strain studied gave the optimal reaction at a still higher pH value than did the klebsiellas of types A and C. A swelling of the capsule was observed regularly only with this organism (Pl. 2, fig. 18).

Table 1. *Non-specific* pH-*dependent capsular reaction of several bacteria with bovine serum diluted* 1/20

Organism	pH of the buffer solution							
	2·0	2·4	2·8	3·2	3·6	4	4·4	4·8
	Observed reaction							
Cryptococcus neoformans	+	+++	+++	−	−	−	−	−
Klebsiella pneumoniae type B	−	++	++	±	−	−	−	−
K. pneumoniae type A, C	(+)	(+)	++	+++	+++	−	−	−
Streptococcus haemolyticus group C	−	±	+	+	±	−	−	−
Diplococcus pneumoniae type III	−	−	±	+	+++	+++	−	−
Bacillus subtilis	−	−	+	++	+++	+	−	−
B. anthracis, 'Vollum'	(±)	(±)	(+)	(+++)	++++	+++	+	−
B. anthracis (vaccine)	(±)	(+)	(+)	(+++)	++++	+++	+	−
B. megaterium	(±)	(±)	(+)	(++)	+++	+++	++	−
Bacillus M	(±)	(+)	(++)	++	+++	++++	+	−

++++ denotes the maximal reaction, the capsule appearing in the phase-contrast microscope as a dark, grey body.

+ denotes a very faint grey capsule.

++ and +++ denote grades between + and ++++.

± denotes the appearance of a faint grey capsule only on some bacteria.

( ) denotes shrinkage of the capsule and increased light scattering of the bacterial bodies.

The chemical nature of the capsular substance of *C. neoformans* was determined by Drouhet, Segretain & Aubert (1950), by Evans & Mehl (1951) and by Evans & Theriault (1953); xylose, mannose and glucuronic acid were found in the purified capsular polysaccharide. *C. neoformans* showed in our studies the lowest pH zone as compared with any other capsulated micro-organisms, with each of the proteins tested. No swelling of the capsule was observed at the optimal pH. Neill, Castillo, Smith & Kapros (1949) also did not observe swelling, when studying the specific capsular reaction of this micro-organism by the Neufeld test. On the contrary, a definite contraction of the capsule was observed, with our method, on the acid side of the reacting pH zone.

In the second group of bacteria, those which produce polypeptide capsules, *B. anthracis* and *B. subtilis* could be separated. Capsular substance of *B. anthracis* was separated from the bacterial polysaccharide by Tomcsik & Szongott (1933); Bodon & Tomcsik (1934) demonstrated that only the polypeptide and not the polysaccharide antibody gives a specific reaction with the capsule of *B. anthracis*. The chemical nature of this capsular substance was cleared up by Ivanovics & Bruckner (1937) who showed that it is a D-glutamic acid polypeptide; these observations were confirmed and extended by Hanby

& Rydon (1946), Hanby, Waley & Watson (1948), Ambrose & Hanby (1949), Housewright & Thorne (1950). Ivanovics & Erdös (1937) isolated the same substance from *B. subtilis*. In the case of *B. anthracis* grown on agar medium with 20% (v/v) atmospheric CO_2 concentration, the non-specific capsular reaction is easily demonstrable with all sera and proteins over a broad zone of pH values (Pl. 1, figs. 2, 3, 5). On the more acid side of this zone (see Table 1) a definite contraction of the capsule was observed (Pl. 1, fig. 6). The bacteria and the contracted capsules showed a peculiar light-scattering which made photography difficult.

Our *B. subtilis* strain had to be incubated only a few hours at 32° in order to show a considerable proportion of capsulated bacilli (Pl. 1, fig. 10). In older cultures the polypeptide is given off as a slimy substance. The pH zone of the non-specific capsular reaction was narrower than with *B. anthracis* and the contracted capsules at acid reaction were hardly visible. A slime reaction, analogous to the non-specific capsular reaction, can be demonstrated occasionally by increasing the concentration of protein. In these cases the pH range at which the capsular and the slime reactions can be demonstrated is the same.

We listed in the third group of capsulated bacteria *B. megaterium* and *Bacillus M* which have a complex capsular composition. As shown in recent studies (Tomcsik, 1951; Tomcsik & Guex-Holzer, 1951, 1952), the polypeptide antibody produced by intravenous injections of killed capsulated *B. anthracis* organisms into rabbits reveals a homogeneously distributed capsular substance in these bacteria (Pl. 2, fig. 14). On the other hand, the homologous specific antibodies, which can be specifically absorbed with the serologically distinct polysaccharides of these two bacteria, induce the visibility of a characteristic capsular structure (Pl. 2, fig. 15), as well as of the cell wall (Tomcsik & Guex-Holzer, 1952). The analysis of these polysaccharides will be reported in another communication. It is, however, interesting in view of the present work that these polysaccharides do not contain uronic acids. The reaction of these bacilli with proteins, that is to say, the non-specific capsular reaction, occurred practically at the same pH range as with the *B. anthracis* strains. The shrinking of the capsule and the light scattering of these bacilli is very pronounced on the more acid side of the reacting zone. In comparing microphotographs, figs. 14 and 15 of Pl. 2, showing the specific capsular reactions with the polypeptide and with the polysaccharide substances of the capsules, with the microphotographs, figs. 11 to 13 of Pl. 2, on the non-specific capsular reactions with proteins, the similarity with the polypeptide reaction is apparent. Later in this paper it will be shown that a purified polysaccharide isolated from these bacteria does not give a precipitation reaction with proteins at an appropriate pH range as the polypeptide does.

Effect of different proteins on the non-specific capsular reaction

The pH zones in which the non-specific capsular reaction occurs with bovine serum remain essentially the same when horse or human serum is used. Human serum has an advantage inasmuch as precipitation and agglutination, which disturbs the microscopic examination of *B. anthracis*, occurs to a lesser degree. *C. neoformans* showed the optimal non-specific capsular reaction with each of these sera at more acid reactions, *B. anthracis* and related bacilli at less acid reaction. The reacting pH zones of the other bacteria covered a range between these two extremes. The zones may shift on using different proteins, but their sequence regarding the different micro-organisms remains unchanged. In consequence only the reactions of *C. neoformans* and *B. anthracis* are summarized in the Tables 2 and 3.

Table 2. *Non-specific* pH-*dependent capsular reaction of* Cryptococcus neoformans *with different proteins*

	pH value of buffer solution used											
	2·0	2·4	2·8	3·2	3·6	4	4·4	5	6	7	8	9
Protein	Reaction observed											
Pepsin	–	–	–	–	–	–	–	–	–	–	–	–
Trypsin	–	–	–	–	–	–	–	–	–	–	–	–
Gelatin	–	–	–	–	–	–	–	–	–	–	–	–
Casein	+	++	+++	+	–	–	–	–	–	–	–	–
Bovine plasma albumin Fr. V.	–	–	–	–	–	–	–	–	–	–	–	–
Horse serum albumin	±	+++	+	–	–	–	–	–	–	–	–	–
Horse serum globulin	–	±	+++	±	–	–	–	–	–	–	–	–
Haemoglobin	±	+	+	++	+++	++	±	–	–	–	–	–
Egg albumin	–	–	–	–	–	–	–	–	–	–	–	–
Egg globulin	–	–	+	–	–	–	–	–	–	–	–	–
Lysozyme	±	±	±	±	±	±	±	±	–	–	–	–
Protamine	–	–	–	–	–	–	–	–	–	–	–	–

Symbols as Table 1.

Table 3. *Non-specific* pH-*dependent capsular reaction of* Bacillus anthracis *with different proteins*

	pH value of buffer solution used											
	2·0	2·4	2·8	3·2	3·6	4	4·4	5	6	7	8	9
Protein	Reaction observed											
Pepsin	–	–	–	–	–	–	–		–	–	–	–
Trypsin	–	–	–	–	–	–	–	–	–	–	–	–
Gelatin	–	(±)	(±)	(±)	+	±	–	–	–	–	–	–
Casein	(±)	(±)	(+)	(+)	++	+++	+	–	–	–	–	–
Bovine plasma albumin Fr. V.	(±)	(±)	(+)	(+)	+	±	–	–	–	–	–	–
Horse serum albumin	±)	(±)	(+)	(++)	+++	+++	–	–	–	–	–	–
Horse serum globulin	(±)	(±)	(+)	(++)	+++	++++	+	–	–	–	–	–
Haemoglobin	(+)	(+)	(+)	(+)	(++)	++	+++	++	–	–	–	–
Egg albumin	(±)	(±)	(+)	(+)	+	±	–	–	–	–	–	–
Egg globulin	(±)	(±)	(+)	(+)	+	±	–	–	–	–	–	–
Lysozyme	–	–	(±)	(±)	(+)	(+)	(+)	+	++	++	–	–
Protamine	±	±	±	±	±	±	±	±	±	±	±	±

Symbols as Table 1.

The concentration of proteins used in the Tables 2 and 3 was 0·05% (w/v). With some of the proteins, as for example casein and haemoglobin, 0·005% (w/v) may elicit a good capsular reaction, whereas with others, such as albumin, less than 0·05% (w/v) gave an indistinct reaction.

Of the proteins studied, only pepsin and trypsin gave a completely negative reaction with each of the capsulated organisms. Gelatin, bovine plasma albumin fraction no. V and egg albumin gave only a weak reaction with the *Bacillus* species. All the other non-basic proteins used reacted well with each of the capsulated micro-organisms.

A correlation between the isoelectric point of the proteins and the shifting of the reacting pH zone was observed with every micro-organism when using horse serum albumin, globulin or haemoglobin. The higher the isoelectric point of the protein, the higher the pH zone at which the optimal non-specific capsular reaction occurred. The behaviour of some of the capsulated organisms towards lysozyme and protamine confirmed this observation. When a reaction occurred at all with these basic proteins, the reacting zone was extended up to weak alkaline reaction. A visible capsular reaction with basic proteins was found only with the *Bacillus* species. A peculiar feature of this reaction was the contraction of the bacterial capsule and the increased refractivity of the bacilli (Pl. 1, fig. 6). Only with the reaction of *B. anthracis* with lysozyme could the capsule be demonstrated with almost normal appearance at pH 6 and 7, when the mixture was incubated several hours at room temperature (Pl. 1, fig. 7). The microscopic examination of *B. megaterium* and *Bacillus M* had to be carried out at this pH very quickly with lysozyme, since these micro-organisms were dissolved very rapidly even at room temperature by a 0·05% (w/v) solution of purified lysozyme.

Another characteristic feature of the reaction with basic proteins and some of the bacteria was in the deformation of the bacterial cell. The strongest deformation was observed in the reaction of protamine on *Bacillus M*. A visibly turbid suspension of these bacilli was killed completely when left for 3 hr. at room temperature, pH 9, with 0·1–0·025% (w/v) protamine solutions. A marked deformation of the bacterial bodies was observed under these circumstances down to 0·006% (w/v) concentration of protamine. The deformation was very much less distinct with micro-organisms not belonging to the genus *Bacillus*.

The correlation of the pH zone of the non-specific reaction with the isoelectric point of the protein is best seen when the upper pH limit giving the strongest reaction is taken into consideration, as can be seen in Table 4. The pH values given in this table were established with electrometric measurements. The data given in Table 4 suggest that these proteins might form electrostatic compounds with the bacterial capsule at an inter-isoelectric pH value. The possibility of the occurrence of other binding forces cannot be excluded since some proteins, e.g. gelatin and ovalbumin, react differently with different capsules. Furthermore, casein, a protein which gives strong capsular reactions, reacts in a pH zone which corresponds rather to the globulin than to the albumin zone, despite the fact that its isoelectric point is identical with that of the albumin. Differences in the reaction towards different micro-organisms are most distinct when using basic proteins such as lysozyme and protamine.

Table 4. *Upper* pH *limit of the capsular reaction with proteins of different isoelectric points*

Protein	Isoelectric point	*Cryptococcus neoformans*	*Bacillus anthracis*
Albumin (horse serum)	4·9	3·0	4·4
Globulin (horse serum)	5·4	3·2	4·6
Haemoglobin	6·8	4·4	5·4
Lysozyme	9·5	*c.* 6	*c.* 7
Protamine	10–12	—	*c.* 8

Precipitation of the capsular substance with proteins at acid reaction

As pointed out previously, several proteins react at pH values on the acid side of their isoelectric points with the capsule of various micro-organisms, thereby making the capsule visible. Depending on the pH value in the reaction zone, the capsule appears either contracted, normal, or in some cases swollen. The reaction is sometimes homogeneous and extends to the whole depth of the capsule, as in the case of albumin (Pl. 1, fig. 2). In other cases, most frequently with casein and with haemoglobin and in lesser degree with globulin, the reaction appears usually as a precipitation at the surface of the capsule (Pl. 1, fig. 5; Pl. 2, fig. 18). Thus microscopic examination gives the impression that the essential feature of the reaction consists of a precipitation.

For the study of the precipitation of capsular substances with proteins *Bacillus M* was selected, because this organism, like *B. megaterium*, contains polypeptide as well as polysaccharide in its capsule (Tomcsik & Guex-Holzer, 1951, 1952). Three fractions were obtained from this organism: a D-glutamic acid polypeptide, a polysaccharide and a mucoprotein. The separation of the D-glutamic acid polypeptide from the polysaccharide was good. It was found that by mixing one volume each of polypeptide and albumin solution with eight volumes of acetate buffer 0·2% polypeptide had to be added to 1–2% albumin to obtain the largest amount of precipitate in a pH zone where the buffer solutions had values from pH 3·3 to 4·2. On increasing the pH of the buffer to 4·4, a turbidity but no precipitation occurred. Less concentrated polypeptide required also 5 to 10 times the quantity of albumin to produce the greatest turbidity; the precipitation was, however, not complete. The albumin used for the acid precipitation of the capsular substances was bovine plasma albumin fraction V. Protamine precipitated the polypeptide even at pH 7.

The purified polysaccharide was isolated by hot-water extraction from the capsulated bacilli; after hydrolysis it showed glucosamine and galactosamine, a trace of phosphorus, but no glutamic or uronic acids. This polysaccharide absorbed the antibodies from the homologous serum, revealing the characteristic capsular structure (Pl. 2, fig. 15) completely, but it gave no precipitation whatsoever with albumin at acid reactions.

From the same organism, extracted with lysozyme, a mucoprotein was obtained which contained a serologically identical polysaccharide; it contained 7·8% nitrogen and 4·1% phosphorus. Uronic acid could not be demonstrated in this preparation. The mucoprotein did not contain any serologically active polypeptide, but it reacted with albumin in a pH zone slightly more acid than that of the polypeptide. A 0·1% (w/v) solution of the mucoprotein was precipitated with 0·5% (w/v) solution of albumin in such a way that in the supernatant fluid neither the mucoprotein nor the albumin could be demonstrated. One analysis, in which higher concentrations of mucoprotein and albumin were used, will be described. Mucoprotein (100 mg.) was dissolved in acetate buffer pH 3·6 (20 ml.). Albumin (250 mg.) dissolved in distilled water (5 ml.) was added to it. An abundant precipitate appeared. The precipitate, redissolved at pH 7, reacted with polysaccharide immune serum at a titre which suggested at most a 50% decrease of polysaccharide activity. The supernatant fluid after centrifugation was deproteinized by Sevag's method (1934). One part was hydrolysed by boiling with 2 N-HCl for 5 hr. Only a trace of reducing substance could be shown after hydrolysis. A precipitin reaction carried out with the unhydrolysed supernatant fluid and polysaccharide antibody suggested an activity decreased to 1/100 of the initial value.

The experiments cited here demonstrate that the polypeptide capsular substance, as well as the mucoprotein of the *Bacillus M*, might produce insoluble salt-like compounds with proteins at a definite pH zone. By changing the pH value, a solution of the compound can easily be obtained. Thus the isolated capsular substance seems to behave in a similar way to the capsule itself. The purified polysaccharide of *Bacillus M* obtained by hot-water extraction, however, did not give a precipitation with albumin at acid reaction. This observation is concordant with our morphological findings, on the basis of the following considerations: (1) the non-specific capsular reaction with proteins (Pl. 2, figs. 11–13) does not reveal the morphologically very characteristic capsular structure; (2) the characteristic capsular structure (Pl. 2, fig. 15) is revealed with an antibody which can be absorbed by purified polysaccharide, or by the muco-protein, but not by the D-glutamic acid polypeptide. At present we are unable to give a satisfactory explanation of why the lysozyme-extracted mucoprotein fraction reacts serologically in a similar way, and with the protein at acid reaction in a different way, as compared with the purified polysaccharide.

DISCUSSION

A non-specific capsular reaction had been demonstrated previously only with pneumococci. Löfström (1944) found that a 'capsular swelling' reaction of *Diplococcus pneumoniae* type XXVII could be elicited by neutral reaction with acute phase protein or with sera obtained from patients in the acute phase of lobar pneumonia or of other febrile illnesses. Calcium ion was essential for this reaction. He showed that the power of the acute phase serum to elicit the capsular reaction went parallel with its ability to give a pneumococcus C-polysaccharide precipitation discovered by Tillett & Francis (1930).

A non-specific 'quellung' of several types of pneumococci at pH 4·0 by a variety of proteins was recognized for the first time by Jacox (1947). We assume that the principle underlying the 'capsular swelling reaction' of pneumococci described by Jacox is identical with the principle of the non-specific capsular reaction described with various micro-organisms in this work; nevertheless, the following differences should be pointed out. Jacox found that the reaction was inhibited by even as low a concentration of sodium chloride as 0·02 M. We generally used 0·85 % saline, corresponding to 0·145 M-sodium chloride, for the solution or dilution of the proteins and for the suspension of bacteria. We observed only in the case of *Cryptococcus neoformans* and the pneumococci that the capsular reaction with albumin was favoured when the NaCl concentration was decreased to 0·07 M. No such effect was observed, however, when using globulin, haemoglobin or other proteins. According to Jacox the non-specific capsular reaction occurs only with pneumococci. He found no reaction with *Klebsiella pneumoniae*, a mucoid *Escherichia coli*, or a mucoid *Streptococcus haemolyticus*, although acid agglutination was observed under these conditions. We could demonstrate the non-specific capsular reaction with all species of capsulated organisms studied, when using the appopriate pH range and with a number of different proteins.

Jacox described the non-specific capsular swelling of pneumococci at pH 4·0. He did not find a difference in the reaction pH zone with albumin or haemoglobin. By using pH intervals of at least 0·4 unit, as we did, it is easy to demonstrate that the reaction pH zones of albumin and of haemoglobin are different and also vary according to the organism tested. To demonstrate the difference between the reaction zones of such proteins as albumin and globulin, whose isoelectric points lay nearer to each other, pH differences of 0·2 should be used. The only protein which, in the work of Jacox, showed a higher pH than 4·0 for eliciting the capsular swelling of pneumococci, was a 3-phosphoglyceraldehyde dehydrogenase preparation. This protein has an isoelectric point at pH 6·55 according to Cori, Slein & Cori (1948). This finding of Jacox fits well with our conception according to which the higher the isoelectric point of the protein, the higher the pH zone at which the non-specific capsular reaction occurs. It would be interesting to see whether the phenomenon observed by Löfström might not also be correlated with a high isoelectric point of the acute phase protein.

Jacox assumes that in the absence of ionized salts, the pneumococcus polysaccharide-protein gel becomes increasingly hydrophilic and therefore capable of enlarging to produce the 'quellung' reaction. We have shown that the non-specific capsular reaction at different pH zones depends as much on the isoelectric point of the proteins used as on the species of organism which produces polysaccharide or polypeptide capsular substance. We assume that the reacting portions of the molecules of the capsular polysaccharides or of the polypeptide are, for instance, uronic acids or D-glutamic acid, leading in an inter-isoelectric zone to the formation of salt-like compounds with proteins;

the reaction is essentially a precipitation. The capsule itself may be contracted, of unchanged volume or, occasionally, swollen, depending on the pH value.

Unfortunately we were not able to carry out electrophoretic determinations on the bacteria used, to determine their isoelectric points. It may be assumed that the isoelectric point of the bacterial surface is lower when the optimal capsular reaction occurs at lower pH. In consequence it should be lower with *Cryptococcus neoformans* and with *Klebsiella pneumoniae* than with *Bacillus anthracis*. We found no data in the literature about the isoelectric point of capsulated *Cryptococcus neoformans* or *Bacillus anthracis*. Harden & Harris (1953) point out that misleading conclusions can result from attempts to interpret information regarding cellular isoelectric points from data obtained by the dye method. They found by micro-electrophoretic measurements that the isoelectric point of *Klebsiella pneumoniae* is 2·48 and of *Bacillus anthracis* 3·1, i.e. the reverse of the values found by dye methods.

This work was carried out with the help of the Rockefeller Foundation.

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EXPLANATION OF PLATES

(All photomicrographs at × 3000, phase-contrast microscope.)

Plate 1

Fig. 1. *B. anthracis*, grown in 20 % CO_2, bovine plasma albumin fraction V. 0·025 %, 1/1280 N-HCl; capsule invisible.

Fig. 2. *B. anthracis* (20 % CO_2) and *Klebsiella pneumoniae*, type B; bovine plasma albumin fraction V. 0·025 %, 1/640 N-HCl. *B. anthracis*: homogeneous capsule; *K. pneumoniae*: capsule invisible.

Fig. 3. *B. anthracis*, 20 % CO_2, *K. pneumoniae*, type B, egg globulin 0·2 %, 1/320 N-HCl. *B. anthracis*: homogeneous capsule; *K. pneumoniae*: capsule with secondary swelling.

Fig. 4. *B. anthracis*, 20 % CO_2, *K. pneumoniae*, type B, bovine serum 1 : 80, 1/240 N-HCl. *B. anthracis*: capsule contracted to a slight degree; *K. pneumoniae*: no capsule swelling.

Fig. 5. *B. anthracis*, 25 % CO_2, casein 0·05 %, 1/640 N-HCl. Capsule reaction: marginal precipitation. Voluminous casein precipitate.

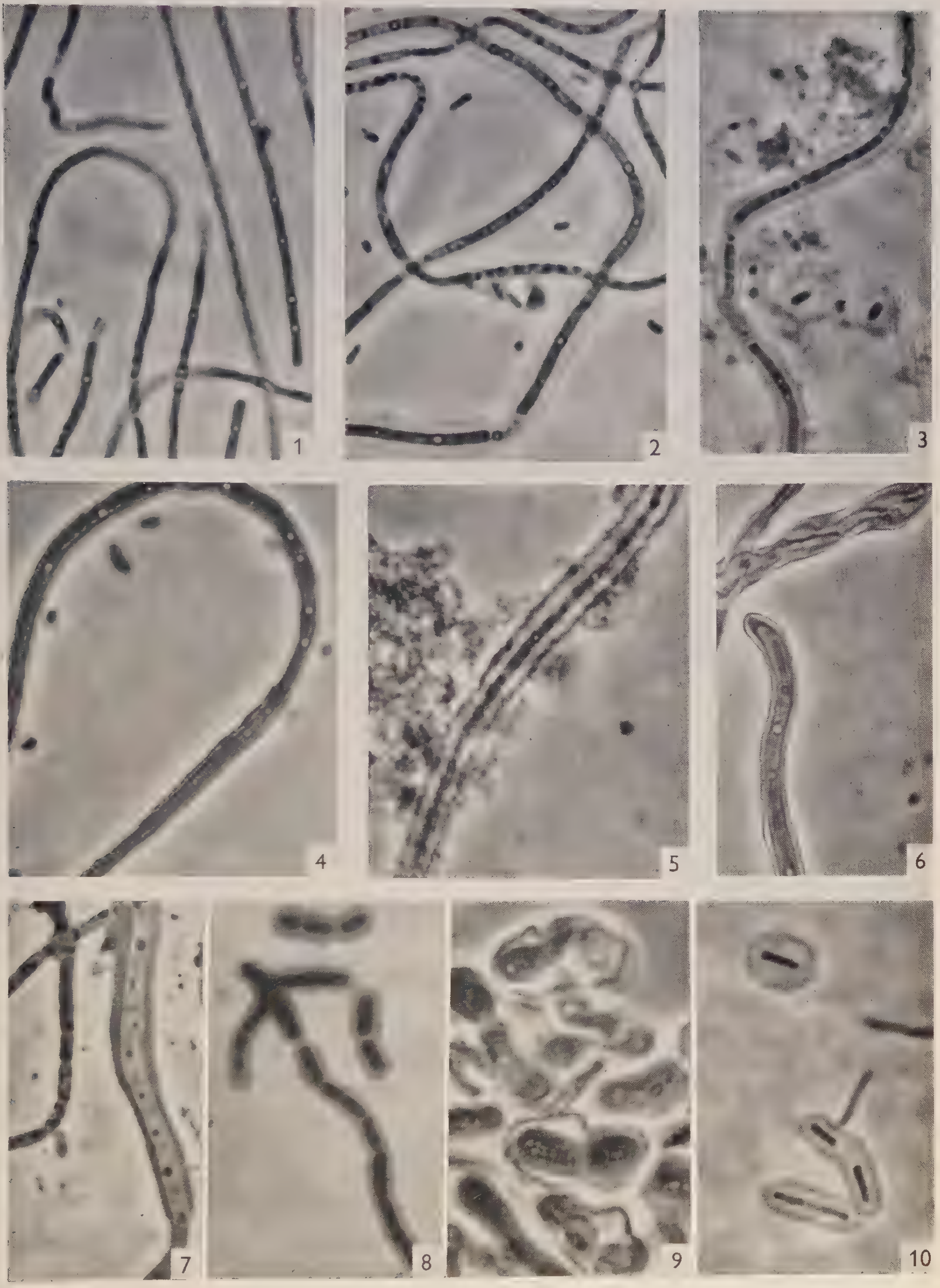
Fig. 6. *B. anthracis*, 25 % CO_2, lysozyme (Mann) 0·2 %, pH of the buffer solution 5·2. Capsule: strong retraction; bacilli: light scattering.

Fig. 7. *B. anthracis*, 20 % CO_2, lysozyme (Alderton) 0·2 %, pH of the buffer solution 9·5. Capsule: homogeneous; bacilli: lysozyme effect.

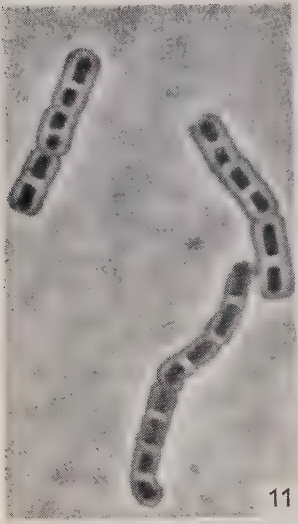
Fig. 8. *B. megaterium*, horse serum 1 : 8, pH 4·1. Capsule: normal, no swelling.

Fig. 9. *B. megaterium*, bovine serum 1 : 8, pH 4·4. Capsule: large swelling and deformation, owing to the lysozyme content of the serum.

Fig. 10. *B. subtilis*, 5 hr. agar-culture, human serum 1 : 20, pH of the buffer solution 3·6. Capsule: very abundant like in indian ink preparation.



J. Tomcsik & S. Guex-Holzer—Reaction of bacterial capsules with proteins. Plate 1



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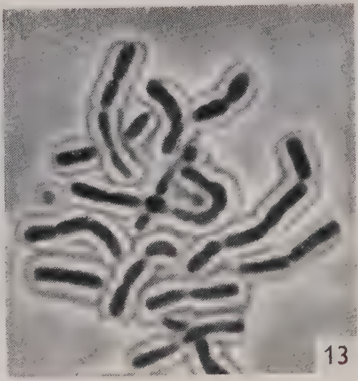

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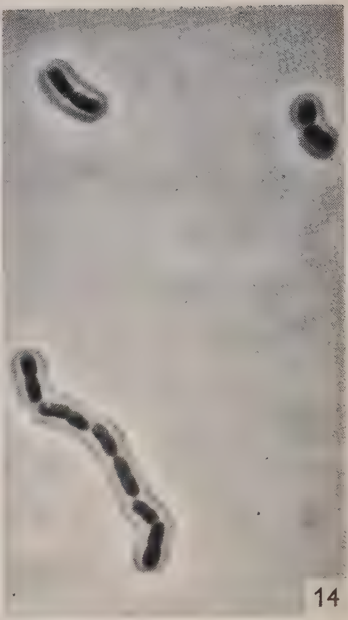

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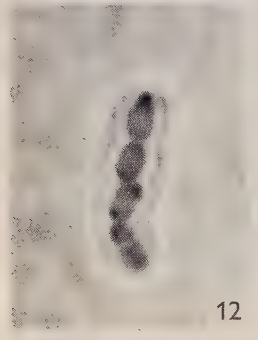

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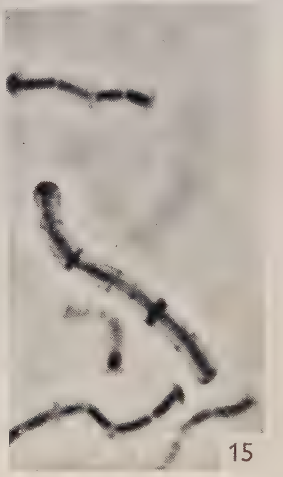

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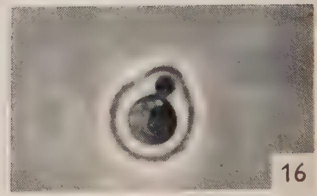

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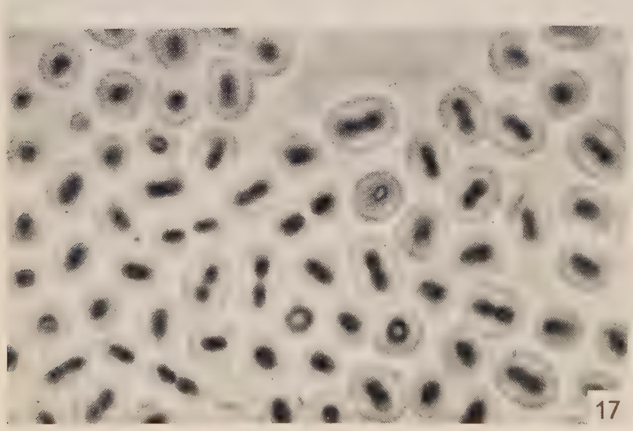

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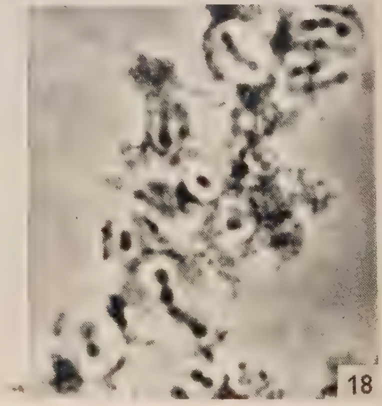

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J. TOMCSIK & S. GUEX-HOLZER—REACTION OF BACTERIAL CAPSULES WITH PROTEINS. PLATE 2

PLATE 2

Fig. 11. *Bacillus M*, bovine serum 1 : 20, 1/400 N-HCl. Capsule with polar depression; bacilli with slight lysozyme effect (1 hr. at room temperature).

Fig. 12. As in Fig. 11, after a few hours at room temperature. Circular structure (it occurs rarely) and polar depression of the capsule, lysozyme deformation of the bacilli.

Fig. 13. *Bacillus M*, bovine serum 1 : 20, 1/300 N-HCl. Agglutinated bacteria, some of them show a polar depression of the capsule.

Fig. 14. *Bacillus M* and D-glutamic acid polypeptide antibody (undiluted) at neutral reaction. The polar portion of the capsule is thin.

Fig. 15. *Bacillus M* and homologous serum, containing polysaccharide antibody (undiluted). Capsule with very marked polar bodies and transverse septa. After absorption of the serum with the purified *Bacillus M* polysaccharide this capsular reaction cannot be elicited. Polypeptide absorption does not alter this reaction.

Fig. 16. *Cryptococcus neoformans*, egg-white 1 : 40, pH of the buffer solution 2·0. The capsule shows the same size as in indian ink preparation.

Fig. 17. *Klebsiella pneumoniae*, type A, human serum 1 : 20, pH of the buffer solution 3·2. Size of the capsule as in wet indian ink preparation.

Fig. 18. Pneumococcus type III, haemoglobin 0·05 %, pH of the buffer solution 3·6. Capsular swelling compared to indian ink preparation is apparent. Precipitation around the capsules.

(*Received* 14 *July* 1953)

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Synergistic and Antagonistic Interactions between Morphogenetically Deficient Variants of the Slime Mould *Dictyostelium discoideum*

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SUMMARY: A group of variant strains of the slime mould, *Dictyostelium discoideum*, was collected, all of which cannot accomplish the complete morphogenetic cycle; instead some stop at intermediate stages. They have been termed 'morphogenetically deficient'. In many cases a mixed population of two deficient strains can achieve synergistic development even to the production of normal fruiting structures with viable spores. The nature and intensity of the synergistic response is a function of the proportions of the partners in the mixture. No recombinant types have been found among the spores obtained from the mixtures. The comparative patterns of response have been ascertained when the deficient stocks are paired in all possible combinations. Finally, an antagonistic interaction between two deficient strains is described.

The slime moulds are exceptional Protista in that they normally undergo a complex sequence of differentiation and morphogenesis leading to the formation of organized multicellular structures. This development becomes apparent after the stationary growth phase is reached and includes the following stages: *Aggregation*, during which the individual myxamoebae move toward central collecting points by means of chemotaxis and under the influence of 'initiator' cells (Bonner, 1947; Sussman & Noel, 1952; Sussman, 1952); *Pseudoplasmodium formation*, wherein the aggregates are transformed into organized, directed bodies of cells which, in some species, migrate over considerable distances (Raper, 1941); *Culmination*, involving the conversion of the pseudoplasmodia into sessile fruiting structures with morphologically differentiated spore, stem, and basal disk cells (Raper & Fennell, 1952). Fig. 1 is a schematic illustration of the cycle.

That this system is open to heritable modification has been demonstrated by the collection of a number of stable variant strains which display aberrant development (Sussman & Sussman, 1953). These strains fall into two phenotypic classes. The first consists of those that follow the normal developmental sequence up to a point after which a deviant pathway is taken, leading to the formation of drastically aberrant fruitings. The second class, termed 'morphogenetically deficient', grows normally but cannot complete the developmental cycle. For example, some stocks cannot begin aggregation (aggregateless), while others (fruitless) can proceed partly or completely through aggregation but stop at intermediate stages before the emergence of the mature fruiting structure.

It has been found that in many cases a mixed population of two of these deficient variants can achieve a level of morphogenesis significantly higher than either partner can accomplish alone, even to the formation of mature fruiting structures with viable spores. In other cases one deficient partner can prevent the other from reaching the stage that it normally attains when alone. The present paper deals with these synergistic and antagonistic interactions and attempts to provide answers to the following questions: (*a*) What types of interaction are observable? (*b*) Do the intensity and the nature of the interactions depend upon the proportions of the paired cell types? (*c*) What is the distribution of phenotype among the spores formed by synergistic development? (*d*) When the deficient stocks are paired in all possible combinations, are distinctive response patterns observable such as might indicate a multiplicity of biochemical alterations leading to the same morphogenetic deficiency?

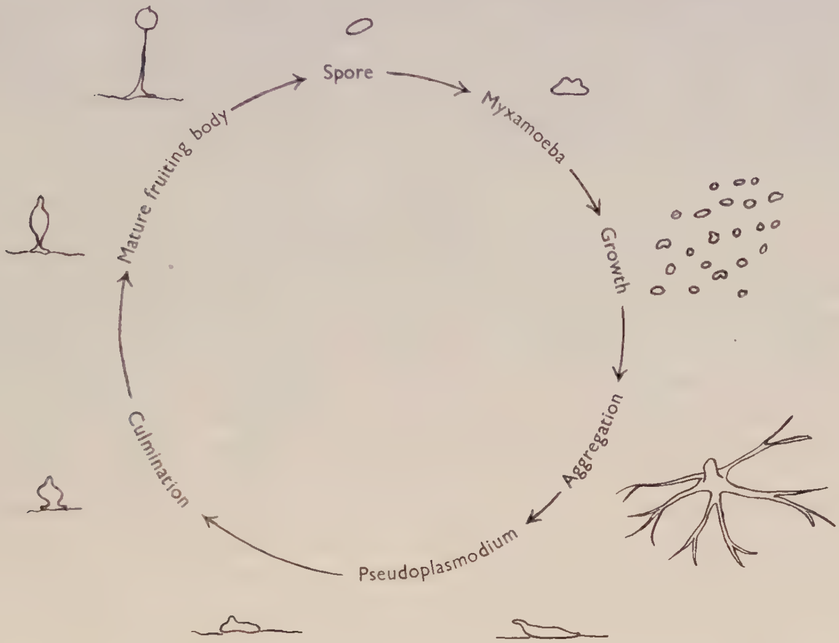

Fig. 1. Schematic diagram of the developmental cycle of *Dictyostelium discoideum*.

METHODS

Organisms. The organism employed in this investigation is representative of *Dictyostelium discoideum* strain NC-4 and variants obtained therefrom.

Medium. The stocks were carried on glucose peptone agar in association with *Aerobacter aerogenes* (Sussman, 1951). For mass platings, a few spore masses or a small amount of myxamoebae were spread on a plate with a few drops of bacterial broth culture and incubated at 22°. After 2 days the myxamoebae had grown to the stationary phase, wiping out the bacterial associate, and were ready for experiment. When colonial growth was required, suitably diluted suspensions were dispensed on plates and spread with bacteria. After 3 days plaques of myxamoebae appeared in the area of bacterial growth.

Assay of developmental ability. The methods used have been described in detail previously (Sussman, 1952). Two-day cultures were harvested and washed by centrifugation and the substantially bacteria-free myxamoebae were suspended in salt solution and counted in a Levy chamber. Suitable dilutions were dispensed in drops on a washed agar distilled water substratum, hereafter designated minimal agar. After the excess fluid had been absorbed by the agar the cells were found to be homogeneously distributed, thus enabling control over the number of cells and the population density. Under these conditions, the viable and total cell counts remain constant, the wild type can aggregate and produce normal fruiting bodies, and the deficient stocks can attain their distinctive levels of development.

RESULTS

Morphogenetic activity of the deficient strains

The variants were obtained by ultraviolet treatment in accordance with procedures given previously (Sussman & Sussman, 1953). In the course of serial passage by clonal platings, some stocks proved to be exceedingly stable while others occasionally threw reverted or mosaic colonies. The extent of development of which these strains were capable were determined in two ways: (1) by examination of the plaques formed on growth medium in the presence of bacteria; (2) by deposition of the washed myxamoebae on minimal agar, after which the plates were incubated and subsequently examined. On these bases the strains fall into four groups. Fig. 2 schematically illustrates the types of development observed on growth medium (column A) and minimal agar (column B). Table 1 contains a listing of the deficient strains according to these groups.

Table 1. *Disposition of the morphogenetically deficient strains among the phenotypic groups*

Group			
I	II	III	IV
Strains			
Agg-53	Agg-55	Fr-4	Fr-1
53-A	58	Fr-5	Fr-2
57	200	Fr-7	Fr-3
59	205	—	Fr-6
204	208	—	—
206	—	—	—

Group I consists of stocks which are completely aggregateless on growth medium and on minimal agar. Group II includes those strains which produce aggregateless plaques on growth medium but which can aggregate and form normal fruiting bodies on minimal agar. The spores when plated back on growth medium provide clones displaying the original aggregateless phenotype. Evidently, the development is inhibited by something in the complete medium or the associated bacteria. Group III consists of those strains which produce fruitless aggregates on minimal agar but which on growth medium form aggregateless plaques. In group IV are the fruitless strains whose morphogenetic activities on growth medium and minimal agar are identical.

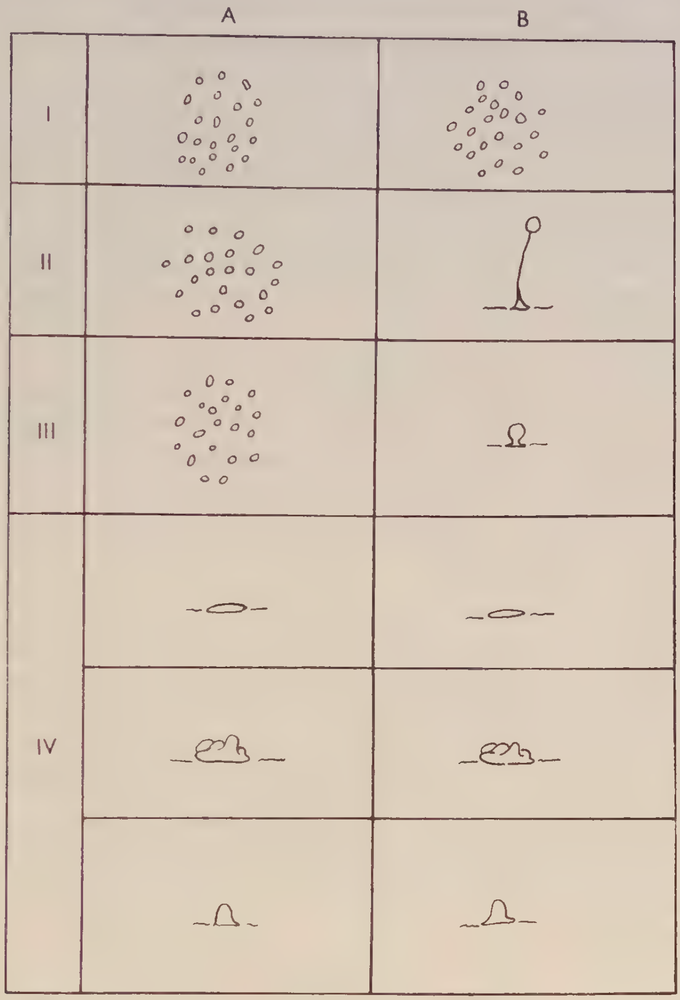

Fig. 2. Developmental capacities of the four phenotypic groups of deficient variants. Column A refers to the maximum development displayed on growth medium with bacteria; column B to that displayed by washed cell suspensions on minimal agar.

The morphogenetic activity of mixtures of deficient strains

The strains were grown and prepared for experiment by procedures described in the Methods section. Appropriate cell mixtures were made and dispensed on minimal agar in replicate 0·01 ml. drops. After 24 and 48 hr. incubation the plates were examined. As stated previously, many of the combinations achieved significantly greater development than either partner alone. Fig. 3

illustrates the general types of synergistic development obtained. These are described below:

Normal or thick-stemmed fruits (*Fig.* 3-*A*). This development is exemplified by the mixture Fr-2 × Agg-59. Fr-2, when alone, aggregated to form irregular cell clumps. Agg-59 did not aggregate at all. Together, they produced normal or thick-stemmed fruiting bodies, depending upon the proportions of the cell types. The spores were normal in appearance and were viable.

Bushy fruits (*Fig.* 3-*B*). An example of this is the mixture Fr-2 × Agg-204. Agg-204 (as well as all the others of the 200 series) was obtained, not from the wild type, but from the 'bushy' variant. The latter aggregated normally but the resulting aggregate, which in the wild type was a smooth cone, became heavily papillated. From each papilla emerged a tiny fruiting body with stalk and spore mass, giving the whole the appearance of a bush. When Fr-2 was mixed with Agg-204, the resulting fruiting structure was typically bushy in appearance.

Flat, migrating aggregates (*Fig.* 3-*C*). This is exemplified by the mixture Fr-1 × Agg-53. Fr-1 alone proceeded partly through aggregation, forming a loose flat circular mass of myxamoebae. Agg-53 is a typical aggregateless strain. When Fr-1 was mixed with small numbers of Agg-53 cells, the flat aggregates which were formed migrated at random over the agar surface precisely as do the normal pseudoplasmodia of the wild type. In contrast to the latter, however, the flat migrating aggregate did not produce a fruiting body at the end of its travels.

Partial culminates (*Fig.* 3-*D*). This is exemplified by the mixture Fr-1 × Fr-4. The mixture reached a higher level of development than did either partner alone, but did not proceed as far as the formation of a mature fruiting structure; instead, morphogenetic activity ceased at an intermediate stage.

The dependence of the synergistic response upon the proportions of the partners

Washed myxamoebae suspensions prepared from the deficient strains were mixed in appropriate dilutions so as to provide a complete range of population ratios. The mixtures were then dispensed on minimal agar in 0·01 ml. drops and these were surveyed after 24 and 48 hr. incubation. In every case the proportions of the deficient stocks played a vital role in determining the type of interaction and its intensity. Data from two representative combinations are given in Tables 2 and 3.

Table 2 shows the results obtained from the mixture Fr-1 × Agg-204. When Fr-1 was greatly in excess, no fruiting occurred and one observed only the fruitless aggregates which Fr-1 normally forms when alone. Where the cell numbers were more nearly equal, extensive fruiting occurred. But when the number of Fr-1 cells was quite small, the construction of fruits was only slightly impeded as long as an excess of Agg-204 cells was maintained.

The mixture of Fr-1 × Agg-53 was capable of two types of synergistic response: (*a*) the emergence of mature fruiting structures from pseudoplasmodia that do not migrate but were otherwise normal; (*b*) the formation of flat, migrating aggregates that did not fruit. From Table 3 it may be seen that a very high ratio of Fr-1 cells to Agg-53 cells allowed only the appearance of the flat migrating aggregates. When that ratio was very low, only mature fruits appeared. Intermediate proportions enabled both types of development to proceed.

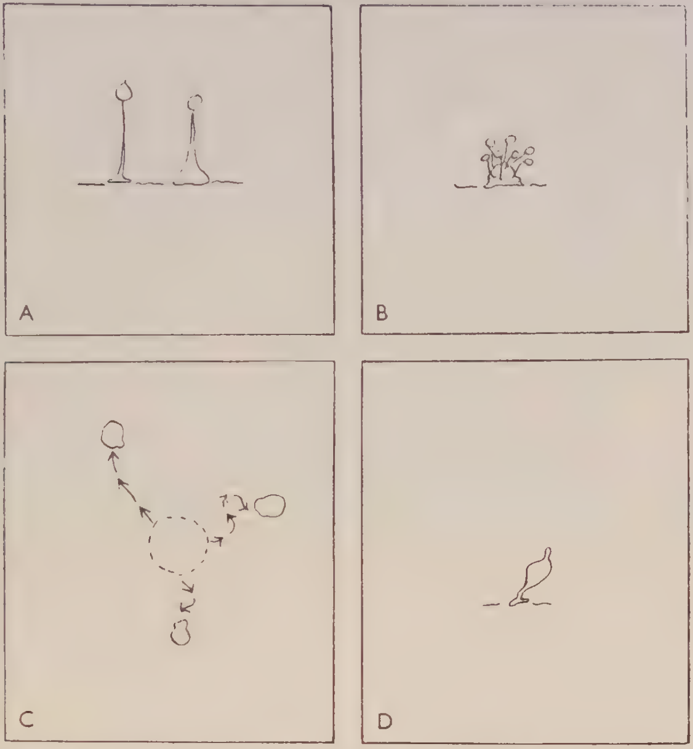

Fig. 3. Schematic illustration of the different types of synergistic development: A—normal and thick-stemmed fruiting bodies; B—bushy fruiting bodies; C—flat migrating aggregates; D—partial culminates.

Table 2. *The dependence of the synergism on the proportion of the cell types. Strain* Fr-1 *combined with strain* Agg-204

Comparative size and number of fruitings scored by number of + signs.

Strain		
Fr-1	Agg-204	
No. cells ($\times 10^{-4}$)		Comparative size and number of fruitings
50	1	0
50	5	0
50	13	+ + +
50	50	+ + + +
13	50	+ + + +
5	50	+ + +
1	50	+ +

Table 3. *The dependence of the synergism on the proportions of the cell types. Strain* Fr-1 *combined with strain* Agg-53

Comparative number of mature fruits and of flat migrating aggregates scored by number of + signs.

Strain Fr-1	 Agg-53		
No. cells ($\times 10^{-4}$)		Mature fruits	Flat migrating aggregates
100	1	0	0
100	10	0	++
100	50	++	+++
100	100	+++	+++
50	100	+++	+++
10	100	+++	0
1	100	++	0

The distribution of phenotype among spores formed during synergistic development

At least two mechanisms might be invoked to explain the synergistic response. One may imagine that the deficient partners exchange metabolic intermediates (syntrophism). The exchange would then enable them to avoid the biochemical blocks that have produced the morphogenetic deficiencies. In this connexion, it may be recalled that Davis (1950) observed a number of such interactions between biochemically deficient strains of *Escherichia coli*, using growth on *minimal* medium as the criterion of syntrophism.

A second possibility is that syngamy may be occurring before the onset of aggregation. Morphogenetic sufficiency could then be acquired by the zygotes themselves or by recombinants from subsequent meioses. In an effort to obtain evidence in support of the second possibility, an analysis of the spores from synergistic fruitings was undertaken. The spores were suspended in salt solution, counted in the Levy chamber, and appropriate dilutions were plated with *A. aerogenes*. After 4–5 days of incubation, the resulting clones were surveyed. Total plaque counts revealed a normal percentage of viability among the spores (efficiency of plating *c.* 75%). The differential counts from three representative experiments out of a total of seven are summarized in Table 4.

Table 4. *Phenotypic analysis of spores from synergistic fruitings*

Combination	No. cells ($\times 10^{-4}$)		No. clones examined	No. aggless clones	% aggless clones
Fr-1 Agg-204	100	100	778	5	0·6
	50	50	1137	11	1·0
	50	100	126	1	0·8
Fr-2 Agg-59	50	50	271	12	4
	50	25	203	4	2
	50	10	443	0	0
Fr-2 Agg-204	50	100	582	45	8
	10	100	86	36	42
	5	100	459	308	67

The plaques proved to be typical in every respect of either of the deficient partners. Not a single wild type or bushy colony or other recombinant type was observed. One might argue that the morphogenetically sufficient zygotes or meiotic products do not appear as spores but instead enter the stalk of the fruiting body. R. Sussman (unpublished data), working with the variants under consideration as well as others in a more extensive attempt to demonstrate sexuality in this genus, has found no evidence to support this contention. Thus it must be concluded that in this instance syngamy is either very rare or non-existent.

Table 4 also indicates that the proportions of the two spore types are determined by the particular combination and by the proportions of the myxamoebae originally present. For example, in the combination Fr-1 × Agg-204, a ratio of 1 of the former to 2 of the latter permitted the appearance of only 0·8 % Agg-204 spores. The same ratio of Fr-2 to Agg-204 allowed 10 times that percentage of Agg-204 spores. Note also that when the ratio of Fr-2 to Agg-204 was decreased from 1:2 to 1:20, the percentage of Agg-204 spores rose from 8 to 67 %.

The comparative patterns of synergistic response

Seven fruitless and six aggregateless strains were paired in all possible combinations to determine which pairs reacted synergistically. For each such combination, a sufficient range of population ratios was employed to be certain of the result. Most of the pairs were tested on two independent occasions. Table 5 summarizes the data. In the case of Agg-57, a complete analysis could not be performed because the strain was lost during the course of the experiments. Table 5 reveals that Agg-53, Agg-53 A, and probably Agg-57 possess identical patterns of response when paired with the other strains (i.e. all three respond positively with Fr-1 and Fr-3 but no others). The same is true of Agg-59 and Agg-204 (i.e. both respond positively with Fr-1, Fr-2, and Fr-3 but no others). The response of Agg-206 is different from those of the aforementioned groups. Finally, all of the seven fruitless stocks displayed different patterns of response. These results indicate that at least three metabolic alterations can lead to the aggregateless condition while at least seven confer the fruitless character.

Table 5. *The comparative response patterns of the deficient strains*

× = synergistic combinations; — = non-synergistic combinations.

	Fr-1	Fr-2	Fr-3	Fr-4	Fr-5	Fr-6	Fr-7	53	53-A	57	59	204	206
Fr-1		—	—	×	—	×	×	×	×	×	×	×	—
Fr-2	—		—	—	—	—	—	—	—	—	×	×	—
Fr-3	—	—		—	×	×	×	×	×	×	×	×	×
Fr-4	×	—	—		×	—	×	—	—		—	—	—
Fr-5	—	—	×	×		—	—	—	—		—	—	—
Fr-6	×	—	×	—	—		—	—	—		—	—	—
Fr-7	×	—	×	×	—	—		—	—		—	—	—
53	×	—	×	—	—	—	—		—	—	—	—	—
53-A	×	—	×	—	—	—	—	—		—	—	—	—
57	×	—	×					—	—		—	—	—
59	×	×	×	—	—	—	—	—	—	—		—	—
204	×	×	×	—	—	—	—	—	—	—	—		—
206	—	—	×	—	—	—	—	—	—	—	—	—	

A note of caution is necessary here. The simplest result from an analysis of this kind would be one in which all strains which possessed different deficiencies reacted positively with each other and only those which possessed the same deficiencies should fail to respond in combination. The situation under discussion is complicated by the fact that many strains which display otherwise different response patterns do not react synergistically when combined. Yet it is true that the second criterion is met since no two strains which display otherwise identical patterns respond positively with each other.

The failure to achieve the simplicity inherent in this type of analysis does not invalidate the conclusions reached. One possible explanation is that the test system is insensitive to the point that it does not permit all possible synergistic relations to be effected. Another is that the accumulation of blocked intermediates or their entrance into side reactions creates inhibitory effects which nullify the potential synergism.

A final observation, not immediately interpretable, is the failure of any two aggregateless strains to respond positively when combined.

Attempts to demonstrate exchange of metabolites

Three kinds of preliminary experiments were performed in an effort to demonstrate exchange; none was successful. The first involved treatment of one deficient stock with a cell-free extract obtained from another deficient strain or the wild type. The second attempted to dissociate the synergistic capacity of either partner from viability by a series of treatments including heating, ultraviolet, and freezing and thawing. In no case could dead cells be shown to react synergistically. The third experimental approach made use of a technique devised by Runyon (1942) who demonstrated the passage of a diffusible chemotactically active substance or substances during aggregation. This worker found that when myxamoebae were allowed to aggregate on agar and underneath a dialysis membrane, other myxamoebae placed on the other side of the membrane become oriented in correspondence with the previously established aggregative centres and streams beneath.

This result was confirmed by the present writer in a similar set of experiments. In addition, it was found that most of the fruitless and at least one of the aggregateless strains so placed above the wild type would so react. But in no case did the development of a deficient stock under these conditions proceed to the formation of fruiting structures. Thus one can be positive that substances were passed from the wild type to the deficient stocks. However, these appear to be substances which normally are exchanged between cells during development and not those which might completely counteract the specific deficiencies.

It is possible that the active intermediates were present in such low concentration and were sufficiently labile that close contact between viable cells was required before the synergistic development could take place. Attempts are under way at present to provide experimental conditions that might conceivably increase the stability.

An example of antagonistic interactions

The strain Agg-208 is one that produces aggregateless plaques on growth medium but aggregates and fruits in washed suspension on minimal agar. Agg-206 is a typical aggregateless stock. When the two were mixed on minimal agar, the ability of Agg-208 to aggregate was partly or completely inhibited, depending on the cell proportions. As shown in Table 6, as little as one Agg-206 to ten Agg-208 cells effected complete inhibition. This antagonism is quite specific. No other aggregateless strain tested could inhibit Agg-208 development. Also Agg-206 did not inhibit the development of the wild type, the bushy variant or Agg-58 whose morphogenetic activity is otherwise identical with that of Agg-208. Preliminary experiments with heat killed Agg-206 cells indicated that their viability was not necessary to promote the inhibition.

Table 6. *The effect of cell proportions on the antagonism between strain* Agg-206 *and strain* Agg-208

Extent of aggregation is scored by number of + signs.

Strain		
Agg-208	Agg-206	
No. cells ($\times 10^{-4}$)		
50	50	0
50	25	0
50	5	0
50	2·5	+
50	0·5	+ + + +
50	0	+ + + +
25	25	0
25	5	0
25	2·5	0
25	0·5	+ +
25	0	+ + +

DISCUSSION

One may envisage at least two opportunities for future investigation that the morphogenetically deficient strains can afford. The first involves an examination of the genetic aspects of the developmental sequence in a manner analogous to that used so successfully with *Neurospora crassa*. However, to achieve that end a clearly defined syngametic system and a method of segregation analysis must be available, as well as a good deal more detailed biochemical information than is now at hand.

A more immediately encouraging prospect would seem to lie in the direction of determining the identities of the metabolites vital to the morphogenetic cycle and to elucidate the biosynthetic pathways that produce them. It is hoped that further investigation will provide an adequate test system for the demonstration of the active materials and their precursors.

Two possibilities were proposed to explain the synergistic response. One involves sexuality and the other the exchange of intermediates in order to avoid the biochemical blocks (syntrophism). A third and less fortunate possibility is that the stocks are deficient because they cannot contribute one or another of the various cell types required for the differentiated assembly of cells. Thus two deficient partners might provide the complete array in concert but not necessarily through exchange of intermediates.

That the developmental sequence does involve passage of at least some substances is clear from the work of Runyon (1942), Bonner (1947), and the results of the membrane experiments reported here. In addition, it has been noted that strains like Fr-1 can aggregate but that none of the myxamoebae therein can be transformed into spores. Yet when mixed with an aggregateless stock, these same cells provide an extremely large proportion of the spore population. It is not readily understandable how this might be contrived except by passage of chemical inductors from the aggregateless cells to the fruitless ones.

This investigation was aided by a grant from the Division of Grants and Fellowships, National Institutes of Health, U.S.A.

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(*Received* 20 *July* 1953)

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The Methyl Red Test in Peptone Media

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SUMMARY: The methyl red (MR) reactions of eighteen strains of coliform bacteria were investigated. Two different media were used, glucose phosphate peptone broth and a liquid defined medium. In the glucose phosphate peptone medium the MR test was found to vary with the brand of peptone used, but the defined inorganic salt + glucose medium gave constant results throughout. After 5 days of incubation at 30° in a glucose phosphate peptone medium twelve substrains selected from each of three strains showed a maximum pH difference of 0·2 unit. When the test strains were inoculated into two batches of this medium made up on different days, pH differences up to 0·6 unit were obtained between the two batches.

The methyl red (MR) test devised by Clark & Lubs (1915) has been used mainly to distinguish between coliform bacteria. The organisms are grown in a medium containing glucose, phosphate and peptone, and after incubation at 30° for 5 days cultures are tested by the addition of a few drops of methyl red solution. *Escherichia coli* cultures show a red colour (MR-positive) and *Klebsiella aerogenes* (syn. *Bacterium aerogenes*, *Aerobacter aerogenes*) a yellow colour (MR-negative). Later, Clark & Lubs (1917) described an improved medium for the test in which all the ingredients were chemically defined. Levine (1916) showed that there was a high negative correlation between the MR and Voges-Proskauer (VP) tests, but Chen & Rettger (1920) described some apparently pure cultures which were persistently MR+, VP+. Taylor, Clegg, Dowson, Hiscox & Richards (1949) maintained that when MR+, VP+ reactions are obtained an impure culture should be suspected since the MR and VP reactions are 'invariably inversely correlated' when the tests are correctly carried out on pure cultures. Fouad & Richards (1952) described a new medium not containing peptone in which MR−, VP+ reactions were given by pure cultures which had been MR+, VP+ in glucose peptone medium.

In our collection we have a series of coliform bacteria which have given different MR reactions in the hands of workers in different laboratories. It was decided to see whether more consistent results could be obtained when these organisms were tested in Fouad & Richards's (FR) defined medium than when tested in glucose peptone medium.

MATERIALS AND METHODS

Dr T. Richards kindly supplied eleven strains that he had found to give MR+, VP+ reactions in glucose phosphate medium, ten of which gave MR−, VP+ reactions in his defined medium. Seven coliform organisms already in the National Collection of Type Cultures, which had given inconsistent MR reactions, were also used. All these eighteen cultures consistently gave positive reactions in the VP test.

Media. The glucose phosphate peptone medium was made up with the following formula (%, w/v): glucose, 0·5; peptone, 0·5; K_2HPO_4, 0·5, in distilled water. Three different brands of peptone were used, and the K_2HPO_4 was stored in a desiccator over $CaCl_2$.

RF glucose salt medium had the formula (%, w/v): glucose, 0·5; NaCl, 0·5; K_2HPO_4, 0·5; KH_2PO_4, 0·2; $(NH_4)H_2PO_4$, 0·2; $MgSO_4.7H_2O$, 0·02; $MnSO_4.4H_2O$, 0·002; $FeCl_3$, 0·00005 (Fouad & Richards, 1952).

Methyl red (MR) *test.* Strains were grown overnight at 30° in peptone water and were subcultured to glucose phosphate peptone medium and to RF glucose salt medium. Cultures were incubated at 30° and a few drops of a solution of 0·04% (w/v) methyl red in ethanol were added at intervals as described in the individual experiments.

Voges-Proskauer (VP) *test.* Cultures were grown at 30° for 5 days in glucose phosphate peptone medium and tested each day by Batty-Smith's (1941) modification of Barritt's (1936) α-naphthol method.

RESULTS

Three batches of glucose phosphate peptone medium, each containing a different brand of peptone, were made up at the same time, and three batches of RF medium were made up by three different assistants.

Table 1. *The effect of different media and duration of incubation on the* MR *test*

Media 4, 5 and 6 = glucose phosphate peptone media; 9 = FR glucose salt medium.

			Days of incubation			
			1	2	3	5
			Result of MR test			
Strain	Medium	Peptone				
8687	4	A	+	−	−	−
	5	B	+	±	−	−
	6	C	−	−	−	−
	9	None	−	−	−	−
8835	4	A	+	±	−	−
	5	B	+	+	+	+
	6	C	+	−	−	−
	9	None	±	−	−	−
8845	4	A	+	+	+	+
	5	B	+	+	+	+
	6	C	+	+	−	−
	9	None	±	−	−	−
418 (control)	4	A	−	−	−	−
	5	B	−	−	−	−
	6	C	−	−	−	−
	9	None	−	−	−	−
8179 (control)	4	A	+	+	+	+
	5	B	+	+	+	+
	6	C	+	+	+	+
	9	None	+	+	+	+

The eighteen test cultures, together with the controls, *E. coli* (NCTC 8179) and *K. aerogenes* (NCTC 418), were inoculated into several tubes of each medium. All cultures were incubated at 30° and on the 1st, 2nd, 3rd and 5th days a tube of each was withdrawn for an MR test. Each batch of glucose phosphate medium gave different results when used for the methyl red test. On the first day, most cultures were MR-positive. Subsequently, some cultures became MR-negative while others remained MR-positive up to the 5th day. Table 1 shows the results of MR tests on three representative strains and the two controls. The three batches of FR medium (only one batch shown in Table 1) behaved more consistently.

The MR reactions after 5 days incubation of all the strains grown in three media containing different peptones are shown in Table 2. All cultures, except the control *E. coli* and NCTC 8696, were MR-negative in FR medium. In this experiment NCTC 8696 gave a negative MR reaction with one batch of peptone medium, but this result was not reproducible.

Table 2. *MR reaction at 5 days in FR medium and in glucose phosphate peptone media containing different brands of peptone*

	Media			
	FR defined	Glucose peptone media		
		Peptones		
		A	B	C
	MR reaction			
Strain				
8696	+	−	+	+
8835	−	−	+	−
8844	−	−	+	−
8845	−	+	+	−
8847	−	−	+	−
8851	−	−	±	−
12 others	−	−	−	−
418 (control)	−	−	−	−
8179 (control)	+	+	+	+

When the strains were tested repeatedly after 5 days incubation in glucose phosphate medium containing peptone B, eight gave consistent results (three MR-positive and five MR-negative) but ten were variable, sometimes being MR-positive and sometimes MR-negative. In tests using FR medium seventeen of the eighteen strains were consistently MR-negative.

Experiments to check the purity of the cultures

To exclude contamination as a cause of inconsistency, three strains that had given the most variable results were replated, and twelve colonies selected from each. The thirty-six substrains so obtained were inoculated into tubes of the same batch of glucose phosphate peptone medium containing bromcresol purple, so that pH values between 5·2 and 6·8 could be estimated by inspection. Unlike methyl red, bromcresol purple was not decolorized by the coliform bacteria. The results of this experiment (Table 3) showed that the twelve substrains of each culture behaved in a similar manner: after 5 days incubation the maximum pH difference between substrains of any culture was 0·2 unit. Thus, strains which in previous experiments showed variable MR reactions in three different batches of peptone medium, produced substrains which reacted consistently when tested in one batch. This indicated that the variable results of earlier experiments were not due to the use of impure cultures; rather were they due to differences in the media used.

Table 3. pH *values attained after 5 days incubation in glucose phosphate peptone medium by substrains of three coliform cultures*

The pH values were estimated by means of bromcresol purple.

	Strain		
	8686	8692	8845
	pH value		
Substrain			
1	6·0	6·0	5·4
2	6·0	6·2	5·4
3	6·0	6·0	5·4
4	6·0	6·2	5·4
5	6·0	6·2	5·4
6	6·0	6·2	5·4
7	6·0	6·0	5·4
8	6·0	6·2	5·4
9	6·0	6·2	5·4
10	6·0	6·0	5·4
11	5·8	6·0	5·4
12	6·0	6·2	5·4

Two batches of glucose phosphate peptone medium containing bromcresol purple were made up on different days; both contained peptone from the same sample. The eighteen strains were grown for 5 days in each batch of medium and the pH values attained are shown in Table 4. Where differences in pH existed, batch 1 tended to show the lower value; a difference of 0·6 unit was shown by three strains.

It is well known that glucose phosphate peptone medium is difficult to sterilize and that when overheated is unsatisfactory in use. Therefore, before sterilization a batch was divided into two portions. Heat was applied normally to one portion and 10 lb./sq.in. pressure was attained in 15 min.; to the second portion the heat was applied more slowly and 40 min. were required to attain the same pressure. When the eighteen coliform cultures were tested in these two portions of medium, only one strain (NCTC 8845) showed a difference of 0·6 unit.

DISCUSSION

It has been shown by many workers that an inverse correlation generally exists between the MR and VP reactions, and as all the strains used in these experiments were VP-positive it was to be expected that they would be MR-negative. However, in glucose phosphate peptone media some of them were

Table 4. pH *values attained on the fifth day by eighteen strains in two different batches of glucose phosphate peptone medium*

The pH values were estimated by means of bromcresol purple

Strain no.	pH of medium Batch 1	 Batch 2	Difference in pH (unit)
8686	5·8	6·0	0·2
8687	5·8	5·8	0
8688	5·4	5·6	0·2
8689	5·8	5·8	0
8690	5·4	5·8	0·4
8691	5·4	5·8	0·4
8692	5·6	6·2	0·6
8693	5·6	6·2	0·6
8694	5·6	5·6	0
8695	5·8	6·2	0·4
8696	5·2	5·4	0·2
8835	5·2	5·8	0·6
8844	5·2	5·4	0·2
8845	5·4	5·4	0
8847	5·4	5·6	0·2
8848	6·0	5·8	0·2
8851	5·4	5·4	0
8866	5·6	5·8	0·2
418 (control)	5·8	6·0	0·2
8179 (control)	5·2	5·4	0·2

MR-positive. Experiments with selected colonies suggested that the strains were pure cultures, but this point could only have been proved by single cell isolations. Wilson, Twigg, Wright, Hendry, Cowell & Maier (1935), who used plating methods and single cell isolations, found that only one of thirty strains giving MR+, VP+ reactions was a mixed culture.

Most of the eighteen strains used here were MR-positive after 24 hr. at 30°, but by the third day, due to the acetoin production, reversal of reaction was occurring. Some strains did not revert within 5 days in certain batches of medium and experiments to discover the reason pointed to the peptone. One strain (NCTC 8696, Richards's R7) remained persistently MR-positive, a result which agreed with Richards's own findings (personal communication).

Of the three brands of peptone used one gave more MR-positive results than the other two, but known differences between successive batches of the same peptone prevent the recommendation or condemnation of any particular brand.

In a medium without peptone, such as FR glucose salt broth, the strains which were initially MR-positive showed an earlier reversal of reaction, and all except NCTC 8696 had become negative by the second day. These experiments suggest that a glucose salt medium is better for the MR test than one containing peptone, and that for coliforms the latter type of medium should be condemned.

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(*Received* 23 *July* 1953)

Jennens, M. G. (1954). *J. gen. Microbiol.* **10**, 127–129.

The Effect of Desiccation on Antigenic Structure

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SUMMARY: Two different organisms were dried in normal rabbit serum and in the presence of specific antibody. Reconstitution of the dried products showed that the presence of antibody did not affect the viability, agglutinability or the absorptive capacity of the bacteria.

Drying by sublimation has become a routine method of preserving bacterial cultures. Although criticism is seldom made in print, several workers who do not themselves dry their cultures suggest that in the drying process there may be a selective killing, or change of unidentified characters, particularly those of an antigenic nature.

In the last four years more than a thousand strains of bacteria have been dried in the National Collection of Type Cultures, and the cultural characters before drying compared with those after drying. The morphological and biochemical characters have never shown any significant change, but some strains have shown an apparent increase in rough-surfaced colonial forms after drying. Most of the strains were dried in filtered horse serum, and it was thought that occasionally a culture might be suspended and dried in the presence of antibody in the so-called normal serum. It seemed unlikely that drying in the presence of antibody would have any effect on the organism, though subsequent growth in the reconstituted serum might increase the proportion of R-forms. Experiments were planned to furnish evidence on this point and cultures were dried in the presence of high titre antibody.

MATERIALS AND METHODS

Strains. A paracolon organism (NCTC 8535, M.R.C. Standards Laboratory strain 3915) with the α-antigen (Stamp & Stone, 1944), and a strain of a salmonella species (NCTC 6759) with the antigenic formula **1, 3, 19**:i↔z, were used.

Antisera. The antisera, prepared in the M.R.C. Standards Laboratory were kindly supplied by Lt-Col. H. J. Bensted. The α-antiserum had a titre of 1/20,000. The salmonella 0 serum had titres of 1/1000 and 1/5000 in two successive bleedings.

Drying of cultures. Strains were grown on Lemco agar and the cells suspended in normal rabbit serum or rabbit antiserum. Volumes of 0·1 ml. were dried in an Edwards's model 3T spin-freeze drying plant, followed by secondary drying for *c.* 18 hr. with P_2O_5 as desiccant. Ampoules were sealed *in vacuo* and stored at room temperature in the dark.

Viable counts. Dried material was reconstituted in broth and normally dilutions were made in broth. Counts were made by the Miles & Misra method (1938).

Agglutination tests. Readings of α-antibody were made after 18 hr. at 50°, of 0 antibody after 18 hr. at 37°.

RESULTS

Effect of antibody on viable count

The paracolon organism was suspended in: (1) normal rabbit serum; (2) rabbit antiserum diluted with normal rabbit serum so that the α-agglutinin titre was 1/1000; (3) rabbit antiserum with an α-agglutinin titre of 1/20,000; (4) broths containing 1 and 7·5 % glucose. Viable counts made before drying and at intervals after drying are shown in Table 1. The differences between the three products dried in serum were not great, and probably not greater than the experimental error. In glucose broth counts were greater both before and after drying, and there was no significant difference between the counts in the two concentrations of glucose. Viable counts are not necessarily counts of single cells; specific antibody may increase the size of bacterial clumps and so diminish the number of colonies. An ampoule of suspension dried in α-antibody was reconstituted in distilled water, and duplicate dilutions were made in broth (containing 0·5 % NaCl) or in distilled water. The absence of electrolyte was expected to inhibit clumping due to a specific antigen-antibody reaction, but in fact the experiment showed a slightly higher count in the series of dilutions made in broth. This experiment indicated that specific antibody was not clumping the bacteria and so artificially decreasing the viable count.

Table 1. *Viable counts of paracolon organisms dried in normal serum and in antiserum*

		Interval (months) after drying				
Suspending fluid	Before drying	0	1	3	6	12
	Viable count × 10^6/ml.					
Normal serum	450	30	9·5	20	10	10
α-Antibody 1/1000	55	45	25	15	10	11
α-Antibody 1/20,000	90	15	2	6·5	3	3
1 % Glucose broth	1400	ND	650	550	400	900
7·5 % Glucose broth	2200	1050	950	1100	750	750

ND = not done.

Effect of antibody on agglutinability

Suspensions were prepared from cultures (1) not dried, (2) dried in normal serum, (3) dried in antibody; each was tested for agglutinability by specific antibody. Paracolon 3915 suspensions were made from cultures that had been dried for 6 months; the salmonella culture was tested soon after drying. The agglutinability of the cultures (Table 2) was not significantly affected by drying and comparable suspensions did not show a difference greater than one tube.

After revival from the dried state the salmonella strain was subcultured rapidly five times and then tested for agglutinability and absorptive capacity (see below); neither property differed from that of the strain before it was dried.

Table 2. *Agglutinability of suspensions made from cultures (a) always grown on media, (b) dried in normal serum, (c) dried in specific antibody*

Suspension from culture	Paracolon	Salmonella
	Agglutination titre	
Not dried	1/10,000	1/5,120
Dried in normal serum	1/10,000	1/5,120
Dried in antibody	1/20,000	1/2,560

Effect of antibody on absorptive capacity

Bulk suspensions were made of paracolon 3915 which had been dried in normal rabbit serum and in rabbit antiserum with an α-antibody titre of 1/20,000. Both suspensions had an absorptiometer opacity equivalent to 95×10^8 organisms/ml. The centrifuged deposit of 10 ml. of each suspension was rubbed up in 5 ml. of α-antiserum diluted 1/15. The mixtures were incubated for 2 hr. at 37°, centrifuged, and the supernatant fluids tested for residual antibody. Absorption was incomplete, each supernatant giving a trace reaction at 1/16 of the diluted serum. Centrifuged deposits of 5 ml. suspension were added to the incompletely absorbed supernatants, and these removed the residual agglutinins.

Absorption of **1, 3, 19** salmonella antiserum by suspensions from cultures that had or had not been dried was similarly carried out in two stages, after which absorption was complete.

DISCUSSION

These experiments did not produce any evidence that the act of drying bacteria in the presence of specific antibody had any significant effect on viability, agglutinability or absorptive capacity. Thus fears that antigenic changes might occur in the drying process, or that a selective killing of sensitive (and antigenically different) cells might change the antigenic pattern of the bacterial population, appear to be groundless.

The antigens studied were of two kinds, a labile and a stable antigen. Neither type of antigen seemed to be damaged by the drying process. It had been assumed by Cowan (1952) that bacteria dried in the presence of specific antibody might be so sensitized that on reconstitution they would agglutinate and so diminish the number of colonies which developed in the viable count, but experiments to show this effect were unsuccessful. Even when bacteria were dried in antiserum with an agglutinating titre of 1/20,000, the decrease in the number of countable colonies was well within the experimental error.

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(*Received* 23 *July* 1953)

Mager, J., Kindler, S. H. & Grossowicz, N. (1954). *J. gen. Microbiol.* **10**, 130–141.

Nutritional Studies with *Clostridium parabotulinum* Type A

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SUMMARY: The nutritional requirements of eight strains of *Clostridium parabotulinum* type A were studied. Five strains required for optimum growth the three vitamins, biotin, thiamine and *p*-aminobenzoic acid, one strain showed additional demands for nicotinic acid and pyridoxin, whilst two non-toxigenic strains required biotin and *p*-aminobenzoic acid. Biotin could be replaced by oleic acid and a number of Tweens, *p*-aminobenzoic by folic acid, citrovorum factor or a mixture of thymine and purines.

All the strains tested showed good growth in a medium containing at least 2·5 % casein hydrolysate (Difco), equivalent to about 0·18 % N. The casein hydrolysate could be replaced by a mixture of amino acids, of which the following nine were essential: tryptophan, threonine, valine, leucine, isoleucine, methionine, arginine, phenylalanine and tyrosine. The last three acids were required in unusually large amounts. Growth in chemically defined medium was followed by a rapid lysis. In spite of optimum growth, the toxin titre in the defined medium was about 50,000 LD_{50} doses/ml., i.e. approximately ten times less than that obtained in complex media.

The cultivation of spore-forming anaerobic bacteria (*Clostridium sporogenes*) in chemically defined media was first attempted by Knight & Fildes (1933). In the following years, defined media for a number of other anaerobic spore-forming species were elaborated (Brown, Wood & Werkman, 1939; McDaniel, Woolley & Peterson, 1939; Weizmann & Rosenfeld, 1939; Rubbo & Gillespie, 1940; Lampen & Peterson, 1944; Feeney, Mueller & Miller, 1943; Bernheimer, 1944; Boyd, Logan & Tytell, 1948; Smith, L. DeSpain & Douglas, 1950). Investigations of the growth requirements of *Cl. botulinum* which started quite early (Hosoya & Kishino, 1925; Burrows, 1933; Fildes, 1935) did not lead to the complete elucidation of the factors involved. Elberg & Meyer (1939), summing up earlier literature and corroborating the results of Fildes & Richardson (1935), concluded that *Cl. botulinum* could be cultivated in a defined medium consisting of ten amino acids, citrate and the unidentified 'sporogenes vitamin' of Knight & Fildes (1933). Pappenheimer (1935) did not succeed in identifying this vitamin; later studies showed it to be not a single chemical entity but to consist of a mixture of several vitamins of the B group. Thus, in the case of *Cl. sporogenes*, Shull & Peterson (1948) were able to replace the 'sporogenes factor' by a mixture of biotin, *p*-aminobenzoic (*p*-AB) and nicotinic acid.

With *Cl. botulinum* Lamanna & Lewis (1946) replaced the 'sporogenes factor' by the following six vitamins: biotin, thiamine, folic acid, calcium pantothenate, choline and pyridoxin. Roessler & Brewer (1946) claimed, on the other hand, that biotin, *p*-AB, nicotinamide, riboflavine and nucleic acid (or its components) were required. As the reports of the last-mentioned groups of investigators are conflicting and of a rather qualitative nature, it was thought worth while to re-investigate the problem.

MATERIALS AND METHODS

Chemicals. Substances used were obtained from the following sources: vitamins from Hoffman LaRoche (Basel) or National Biochemicals Corporation (NBC).

Citrovorum Factor (CF): Leucovorin calcium salt, kindly supplied by Dr H. P. Broquist of the Lederle Laboratories. Biocytin, through the courtesy of Dr L. D. Wright of Sharp and Dohme Co., Michigan. The synthetic pyrimidine moiety of thiamine (2-methyl-5-ethoxymethyl-6-aminopyrimidine) and the synthetic thiazole moiety of thiamine (4-methyl-5-hydroxyethyl thiazole) from Merck. Casein hydrolysate (CH), Casamino acids Vitamin Free (Difco) or Casein Hydrolysate Vitamin Free 10% soln. (NBC). Sodium mercaptoacetate (thioglycollate; NBC). All other chemicals were of A.R. grade.

Glassware. All glassware was cleaned with chromic + sulphuric acid mixture, washed with running tap water, and finally rinsed three times with twice glass-distilled water.

Media. All media were derived from the following basal medium, which contained, for each litre; glucose, 5 g.; tryptophan, 50 mg.; cysteine, 250 mg.; Na mercaptoacetate, 500 mg.; salts A soln. (Snell, Strong & Peterson, 1937), 10 ml.; salts B (Snell *et al.* 1937), 1·0 ml.; the pH was adjusted to 7·4. Double-strength medium was dispensed into matched test tubes plugged with cotton-wool. Wrapping the cotton plugs with cellophan or replacing them by metal caps did not affect the results.

Sterilization was effected by autoclaving for 15 min. at 15 lb./sq.in. Glucose, vitamins and other substances were sterilized by Seitz filtration and added aseptically to the basal medium. The volume in each tube was then made up to 10 ml. with double-distilled water. The tubes were heated in a boiling-water bath for 10 min., cooled to room temperature and inoculated immediately. All experiments were run in duplicate.

Strains and inocula. Experiments were carried out with the following strains: strain 1, National Collection of Type Cultures, NCTC 3811 (Hall); strain 2, obtained from the Hebrew University, Jerusalem; strain 3, NCTC 7272 (Porton); strain 4, American Type Culture Collection (ATCC) 441; strain 5, ATCC 687; strain 6, ATCC 3502 (Hall); strain 7, ATCC 7948; strain 8, NCTC 2916. Stock cultures were carried in cooked meat broth or in Brewer's anaerobic medium (Difco). Inoculations into the defined medium were made from 24 hr. cultures; 0·1 ml. was added to each tube. In some experiments the inoculum was washed 3 times with saline containing 0·05% (w/v) Na mercaptoacetate and diluted 1/100 with the same solution. Usually, however, washing was omitted because of its harmful effect on viability. Carry-over of growth factors could be eliminated by serial transfers. In a few instances a washed suspension of spores was used as inoculum. Spore suspensions were obtained by growing the organisms in brain-heart medium (Difco) for 5 days, and heating the culture at 70° for 30 min.; the spores were then centrifuged down and washed with mercaptoacetate-saline.

Incubation. The tubes after inoculation were incubated at 34° in anaerobic jars (McIntosh & Fildes), evacuated by a vacuum pump and refilled with hydrogen. In some experiments 0·01 % sodium bicarbonate was added to the medium to ensure adequate supply of CO_2, but under our conditions only a slight, if any, effect of this addition was noticed.

Estimation of growth. Growth was usually estimated by measurement of turbidity with a junior Coleman Spectrophotometer at 450 mμ. and expressed either in terms of percentage transmission or as optical density. In some experiments the turbidimetric results were correlated with viable counts. The counts were made in Brewer's anaerobic agar (Baltimore Biological Laboratories, Maryland, U.S.A.) and the Petri dishes incubated in anaerobic jars.

Assay of toxin. Toxin production was usually tested after growth for 5 days in a medium similar to that used by Lewis & Hill (1947). Results were calculated in terms of LD_{50} according to the method of Reed & Muench (1938).

RESULTS

Nitrogen requirement

Attempts to grow *Cl. parabotulinum* type A in media containing 0·5 % (w/v) casein hydrolysate, as recommended by Lamanna & Lewis (1946), were unsuccessful even when the number of growth factors added included all the known B complex. Growth if any was irregular and scanty. In order to test the possibility that lack of growth was due to the inadequate supply of some amino acids, the effect of increasing concentrations of casein hydrolysate was tried. The experiments showed that optimal growth of all strains could be obtained only at a level of 2·5 % (w/v) casein hydrolysate, providing 17·5 mg. N/10 ml. medium. This concentration of casein hydrolysate was therefore used in all further work. Since these results suggested that some amino-acids might be required in unusually large amounts, we tried the effect of adding excess of amino-acids, in groups of 1–19, to the basal medium containing 0·5 % casein hydrolysate. Of the nineteen amino-acids tested, the combination arginine (30 mg./10 ml.) and phenylalanine (20 mg./10 ml.) and tyrosine (2·5 mg./10 ml.) yielded growth exceeding that in a medium containing 2·5 % casein hydrolysate. Although tyrosine and phenylalanine substituted for each other to some extent, both were essential and maximum growth was obtained only when both were present in relatively large concentrations. Arginine could be replaced more or less adequately by ornithine or lysine.

Amino acid requirements

In order to arrive at a fully defined medium, casein hydrolysate was replaced by the following mixture of nineteen amino acids: L-arginine, DL-phenylalanine, L-tyrosine, L-tryptophan, DL-leucine, DL-isoleucine, DL-valine, DL-threonine, DL-methionine, L-histidine, DL-lysine, DL-alanine, L-proline, DL-serine, DL-glutamic acid, DL-aspartic acid, L-hydroxyproline, glycine and L-cysteine. The concentrations used are shown in Table 1. Further experiments showed the following nine amino acids to be essential: L-arginine, DL-phenylalaline, L-tyrosine, DL-leucine, DL-isoleucine, DL-valine, L-tryptophan, DL-threonine and DL-methionine (Table 1). These findings are in agreement with the results of Roessler & Brewer (1946).

Table 1. *Amino acid requirements of* Cl. parabotulinum *type A*

Representative values for strains 1 to 8.

Basal medium with biotin, thiamine and *p*-AB added. *Nineteen amino acids* (mg./10 ml.): L-arginine 30, DL-phenylalanine 20, L-tyrosine 2·5, DL-valine 20, DL-leucine 15, DL-isoleucine 5, L-tryptophan 0·5, DL-threonine 10, DL-methionine 6, L-proline 4·5, L-hydroxyproline 1, L-histidine 2, DL-glutamic acid 10, DL-aspartic acid 9, DL-lysine 12, DL-alanine 4·2, DL-serine 10, L-cysteine 2·5, glycine 1.

Nine amino acids (mg./10 ml.): L-arginine 30, DL-phenylalanine 20, L-tyrosine 2·5, DL-valine 20, DL-leucine 15, DL-isoleucine 5, L-tryptophan 0·5, DL-threonine 10, DL-methionine 6.

N source	% transmission after 24 hr.
19 Amino acids	23
9 Amino acids	32
Amino acid omitted from mixture of 9	
L-arginine	100
DL-phenylalanine	88
L-tyrosine	100
DL-valine	90
DL-leucine	55
DL-isoleucine	76
L-tryptophan	94
DL-threonine	84
DL-methionine	80
DL-leucine and DL-isoleucine	100

The mixture of nine amino acids gave consistently good growth, which was, however, somewhat inferior to that obtained with the mixture of nineteen amino acids. It seems, therefore, that the remaining amino-acids had some adjuvant effect. Methionine could be partially replaced by cysteine, but full growth was not obtained when cysteine alone was present. Leucine and isoleucine substituted for each other to some extent.

Vitamin requirements

To determine the vitamins required for optimum growth, one vitamin at a time was omitted from an otherwise complete medium containing initially six vitamins. The results of these experiments are summarized in Table 2.

When 'Casamino acids' (Difco) was used as a source of nitrogen, it appeared that biotin and thiamine were the only essential growth factors. Slight growth was consistently obtained with biotin as the only vitamin added; *p*-AB seemed only to be stimulatory. On the other hand, when the source of amino acids was a casein hydrolysate prepared in our laboratory (by autoclaving casein with sulphuric acid and removal of vitamins by adsorption with 'Norit A' charcoal) an absolute requirement for *p*-AB was found. It appeared therefore that Difco casein hydrolysate contained small amounts of *p*-AB or a substance possessing similar biological activity. The essential requirement for these three vitamins was further confirmed when a completely defined medium containing a mixture of amino acids in place of casein hydrolysate was used (Table 2).

Table 2. *Effect of vitamins on growth of* Cl. botulinum *type A*

C.H. (Difco) = Casamino acids Difco 'vitamin-free'.

CHS = Sulphuric acid hydrolysate of casein, 'Norit' adsorbed (17·5 mg. N/10 ml.).

Amino acid mixture: nine amino acids, see Table 1.

Sixteen vitamins: (μg./10 ml.) Biotin, 0·005; thiamine, 4; folic acid, 0·1; choline, 25; Ca-pantothenate, 10; pyridoxin, 5; pyridoxamine, 5; pyridoxal HCl, 5; pyridoxal phosphate, 5; nicotinic acid, 10; nicotinamide, 10; riboflavin, 5; inositol, 400; citrovorum factor, 0·1; *p*-AB, 0·1; B_{12}, 0·5.

Six vitamins: (μg./10 ml.) biotin, 0·005; thiamine, 4; folic acid, 0·1; choline, 25; Ca-pantothenate, 10; pyridoxine, 5.

Five vitamins: (μg./10 ml.) *p*-AB, 0·1; nicotinamide, 10; riboflavin, 5; yeast nucleic acid, 200; biotin, 0·005.

		Times of incubation			
		20 hr.	38 hr.	46 hr.	84 hr.
N source	Vitamins added to basal medium	% Transmission			
C.H. (Difco) 2·5 % w/v	16 vitamins	55	32	45	82
	6 vitamins	90	34	37	60
	5 vitamins	92	90	87	85
	Biotin, thiamine, *p*-AB	71	34	37	61
	Biotin, thiamine	92	72	60	40
	Biotin, *p*-AB	92	90	90	84
	Biotin, Ca-pantothenate	98	90	86	84
	Thiamine, *p*-AB	100	100	100	100
	Biotin	98	85	82	82
CHS	Biotin	97	—	—	70
	Biotin, thiamine	96	—	—	65
	Biotin, thiamine, *p*-AB	35	—	—	87
Amino acid mixture	Biotin	90	—	—	85
	Biotin, thiamine	94	—	—	80
	Biotin, thiamine, *p*-AB	37	—	—	82
	Biotin, *p*-AB	92	—	—	84

The growth response to each essential vitamin was determined as illustrated in Figs. 1 and 2. No change in nutritional or other properties of the organism was observed even after fifty serial transfers in the defined medium.

p-AB, citrovorum factor and folic acid were interchangeable (Fig. 2). Folic acid appeared to be the least active compound. This observation would imply that folic acid was not an intermediary in the biosynthesis of the final co-factor from *p*-AB (cf. Woods, 1952). Once an absolute requirement for the three vitamins had been established, their substitution was attempted by substances known to have similar physiological action. From Table 3 it can be seen that the requirement for biotin could be met by sodium oleate and several 'Tweens'. In this respect *Cl. botulinum* behaved similarly to *Cl. sporogenes* as observed by Shull, Thoma & Peterson (1949). The thiazole moiety of thiamine was as effective as the whole molecule, indicating that this organism is capable of synthesizing the vitamin from the thiazole derivative.

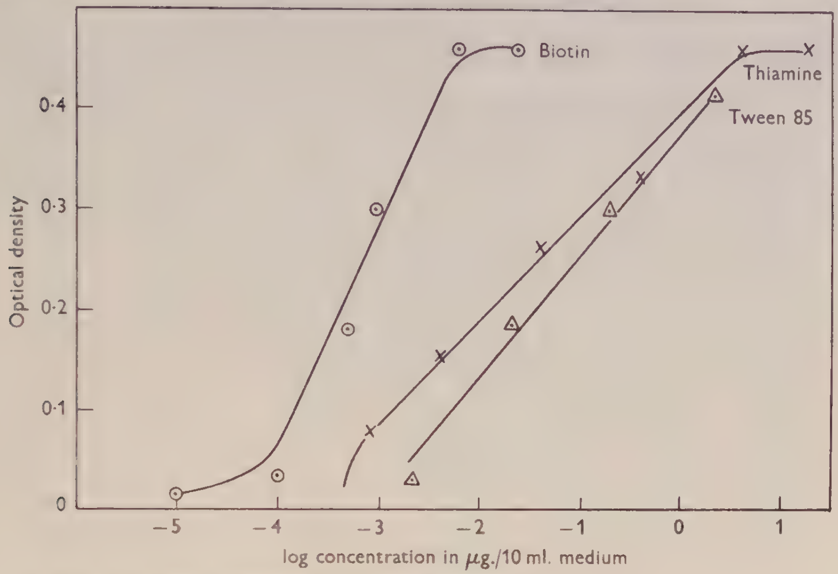

Fig. 1. Growth response of *Cl. parabotulinum* type A (strain 1) to various concentrations of biotin, thiamine and Tween 85.

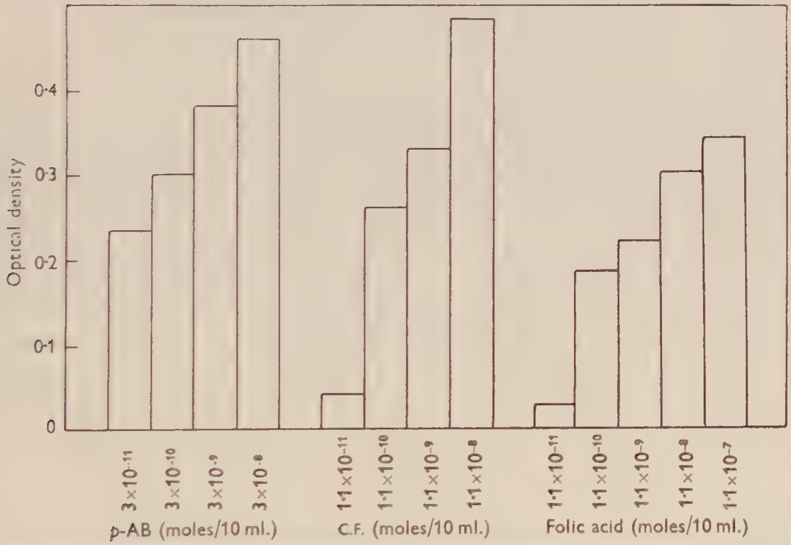

Fig. 2. Comparison of growth responses of *Cl. parabotulinum* type A (strain 1) to various concentrations of *p*-AB, citrovorum factor (CF) and folic acid.

The fact that *p*-AB could be replaced not only by folic acid and the citrovorum factor, but also by a mixture of adenine and thymine, is compatible with the view that *p*-AB serves as a precursor of a coenzyme involved in the synthesis of the latter substances (cf. Woods, 1952).

Table 3. *Replacement of vitamins by substances of similar physiological activity in nutrition of strains* 1 *and* 2

Sulphuric acid hydrolysate of casein, 'Norit'-adsorbed (17·5 mg. N/10 ml.), was added to the basal medium.

Vitamin added to basal medium (μg./10 ml.)	% Transmission after 38 hr.
Biotin, 0·005; thiamine, 4; *p*-AB, 0·1	43
Biotin, 0·005; thiamine, 4; folic acid, 0·1	41
Biotin, 0·005; thiamine, 4; citrovorum factor, 0·1	35
Biotin, 0·005; thiamine, 4; B_{12}, 0·1	85
Biotin, 0·005; thiamine, 4; thymine, 200	78
Biotin, 0·005; thiamine, 4; thymine, 200 + adenine, 200	45
Biotin, 0·005; thiamine, 4; thymidine, 200	62
Biotin, 0·005; thiamine, 4; thymidine, 200 + adenine, 200	55
Biotin, 0·005; thiamine, 4; yeast nucleic acid, 200	55
Biotin, 0·005; thiamine, 4; thymus nucleic acid, 200	90
Biotin, 0·005; thiazole of thiamine, 2, *p*-AB, 0·1	45
Biotin, 0·005; pyrimidine of thiamine, 2; *p*-AB, 0·1	95
Na Oleate, 1000; thiamine, 4; *p*-AB, 0·1	52
Tween 80, 2000; thiamine, 4; *p*-AB, 0·1	55
Tween 81, 2000; thiamine, 4; *p*-AB, 0·1	50
Tween 85, 2000; thiamine, 4; *p*-AB, 0·1	53
Tween 40, 2000; thiamine, 4; *p*-AB, 0·1	95
Tween 60, 2000; thiamine, 4; *p*-AB, 0·1	93
Pimelic acid, 10; thiamine, 4; *p*-AB, 0·1	100
Biocytin, 0·01; thiamine, 4; *p*-AB, 0·1	100

Comparison of the vitamin requirements of various strains of Cl. parabotulinum *type A*

From the results summarized in Table 4 it can be seen, that of the eight strains examined, five showed identical requirements for the three vitamins biotin, thiamine and *p*-AB. Strain 8 was more exacting and required in addition pyridoxin and nicotinic acid. The identity of the toxin of strain 8 with the serological type A was confirmed by the positive outcome of a neutralization test with a specific anti-A serum obtained from the Pasteur Institute, Garches. Strains 4 and 5 did not require thiamine; furthermore, they did not produce any toxin and differed morphologically from the other strains; their growth on solid media was of the rough (R) rhizoid type resembling closely the growth of *Cl. sporogenes.* As there is no certain way of differentiating between *Cl. sporogenes* and non-toxigenic strains of *Cl. botulinum* by the usual cultural or biochemical methods, it cannot be decided whether these strains represent non-toxigenic mutants of *Cl. parabotulinum* type A or are identical with *Cl. sporogenes* and were incorrectly classified as *Cl. botulinum* type A. In this connexion it should be recalled that the usual growth factors of most strains of *Cl. sporogenes* are biotin and *p*-AB, while nicotinic acid is only stimulatory (Shull & Peterson, 1948; Mager, unpublished results).

The claim of Lamanna & Lewis (1946) that thiamine and choline can be replaced by calcium pantothenate was not borne out by our results. As can be seen from Table 2, choline had no effect at all on growth, whereas (Table 4) pantothenate did not replace thiamine for any of the strains tested.

Table 4. *Comparison of various strains of* Cl. parabotulinum *type A in respect of vitamin requirements*

Sulphuric acid hydrolysate of casein, 'Norit'-adsorbed (17·5 mg. N/10 ml.), was added to basal medium.

Vitamins (μg./10 ml.): biotin, 0·005; thiamine, 4; *p*-AB, 0·1; calcium pantothenate, 10; pyridoxin, 10; nicotinic acid, 10.

Strain	LD_{50}/ml.	Vitamins added to basal medium	% Transmission after 25 hr.
1	500,000	Biotin	96
		Biotin, *p*-AB	97
		Biotin, *p*-AB, thiamine	37
		Biotin, *p*-AB, Ca-pantothenate	96
2	500,000	Biotin	94
		Biotin, *p*-AB	94
		Biotin, *p*-AB, thiamine	42
		Biotin, *p*-AB, Ca-pantothenate	95
3	500,000	Biotin	92
		Biotin, *p*-AB	90
		Biotin, *p*-AB, thiamine	45
		Biotin, *p*-AB, Ca-pantothenate	89
4	0	Biotin	76
		Biotin, *p*-AB	42
		Biotin, *p*-AB, thiamine	40
		Biotin, *p*-AB, Ca-pantothenate	42
5	0	Biotin	82
		Biotin, *p*-AB	41
		Biotin, *p*-AB, thiamine	41
		Biotin, *p*-AB, Ca-pantothenate	43
6	300,000	Biotin	94
		Biotin, *p*-AB	95
		Biotin, *p*-AB, thiamine	55
		Biotin, *p*-AB, Ca-pantothenate	94
7	500,000	Biotin	93
		Biotin, *p*-AB	95
		Biotin, *p*-AB, thiamine	44
		Biotin, *p*-AB, Ca-pantothenate	92
8	200,000	Biotin	95
		Biotin, *p*-AB	98
		Biotin, *p*-AB, thiamine	85
		Biotin, *p*-AB, Ca-pantothenate	95
		Biotin, *p*-AB, thiamine, pyridoxin, nicotinic acid	31

Size of inoculum

In order to test the nutritional adequacy of the defined medium, we determined the minimum number of cells capable of initiating growth. Viable counts indicated that an inoculum containing as few as ten vegetative cells was sufficient to start growth, provided that dilutions were made in the basal medium; when saline was used as diluent the viability of the inoculum was considerably decreased. This threshold value compares favourably with that obtained with more complex media. Similar results were obtained with washed spores.

Effect of glucose

In a medium containing 2·5% casein hydrolysate supplemented with arginine, phenylalanine and tyrosine, omission of glucose resulted in only slightly inferior growth. On the other hand, growth in the unsupplemented medium was much poorer when glucose was absent.

Mineral requirements

Marked depression of growth was observed when depletion of trace minerals was effected by treating the medium with Al_2O_3 as described by Donald, Passey & Swaby (1952). Full growth was restored when mixtures of minerals were added; the effects are shown in Table 5.

Table 5. *Effect of salts on growth of* Cl. parabotulinum *type A* (*strains* 1 *and* 2)

2·5% (w/v) C.H. (Difco) added to basal medium, from which salts were omitted. This medium as well as glucose soln. was treated with Al_2O_3 in order to remove traces of salts.

Vitamins added (μg./10 ml.): biotin, 0·005; thiamine, 4; *p*-AB, 0·1.

Salts A (Snell *et al.* 1937); Salts B (Snell *et al.* 1937); Salts C (μg./10 ml.): $ZnSO_4.7H_2O$, 80; $CuSO_4.5H_2O$, 45; $CoCl_2$, 90; $CaCl_2.2H_2O$, 60; $Na_2B_4O_7.10H_2O$, 140; $(NH_4)_2MoO_4$, 25.

Salts added	% Transmission after 48 hr.
—	87
Salts A	73
Salts A and B	59
Salts A, B and C	41

Autolysis

Cultures of *Cl. parabotulinum* in defined media exhibited a tendency to lyse rapidly. The autolysis was not related to sporulation and could be so pronounced as to result in complete sterilization of the culture. This property complicated seriously the estimation of maximum growth and made it necessary to determine growth curves as a function of time, for every set of conditions.

From Figs. 3 and 4 it can be deduced that most rapid lysis occurred under optimal conditions of growth, whereas when the vitamin supply was suboptimal the rate of autolysis was much slower. It appears, therefore, that the formation of autolytic enzymes depends on an adequate supply of certain nutrilites. As shown in Fig. 4, the rate of autolysis increased also with the rise of the initial pH value of the medium, from pH 6·5 to 7·8. Here again the more rapid the growth the steeper the decline caused by autolysis.

Toxin production

The production of toxin in the defined medium was relatively low as compared with yields in more complex media. For instance the usual titre in the medium of Lewis & Hill (1947) for the majority of strains of type A

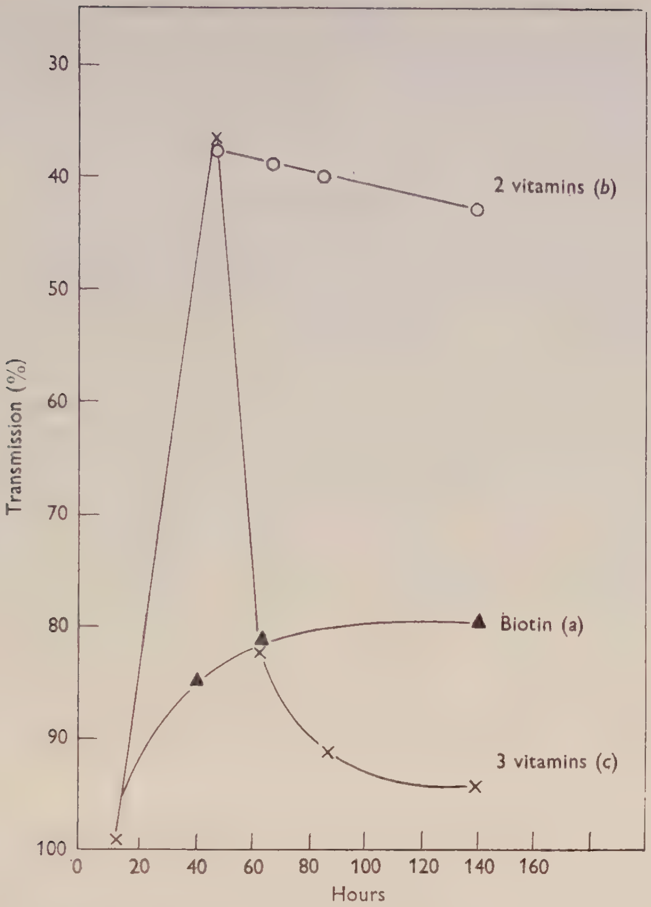

Fig. 3. Growth and lysis of *Cl. parabotulinum* type A (strain 1) in media containing: (*a*) biotin; (*b*) biotin and thiamine; (*c*) biotin, thiamine and *p*-AB.

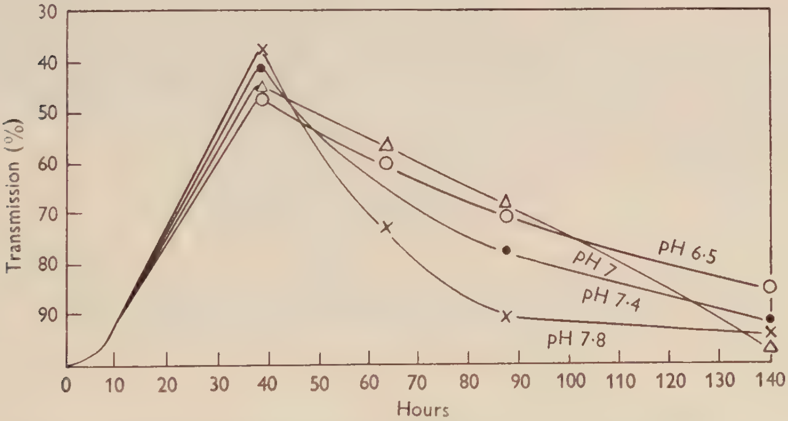

Fig. 4. Effect of the initial pH of the defined medium on the rate of growth and lysis of *Cl. parabotulinum* type A (strain 1).

varied between 500,000 LD_{50} doses/ml. and 1,500,000 LD_{50} doses/ml., whereas in our defined medium the maximum titre obtained was usually below 50,000 LD_{50} doses/ml. Evidently factors different from those that determine growth regulate the yield of toxin in the culture medium. An account of an investigation of the nature of these factors will form the subject of a separate communication.

DISCUSSION

Our results differ in many points from those reported by Lamanna & Lewis (1946), and by Roessler & Brewer (1946). The preliminary nature of the reports of these authors does not permit a thorough discussion of the reasons for these discrepancies. It seems unlikely, however, that strain variations could account for all the differences noted, as none of the eight strains examined by us conformed in their growth requirements to those described by the above authors. In this connexion it should be pointed out that *Cl. parabotulinum* type A, in the light of our results, appears to be a rather homogeneous group since out of six toxin-producing strains, five showed identical requirements for these vitamins and only one strain differed in demanding two additional vitamins. Of particular interest is the fact that, whereas Roessler & Brewer (1946) found *Cl. botulinum* type A not to require thiamine, all the toxigenic strains studied by us failed to grow in its absence. On the other hand, two strains which did not depend on thiamine for growth proved to be completely non-toxigenic. However, more work with a larger collection of strains will be needed before it can be decided whether a requirement for thiamine offers a valid criterion for differentiating toxigenic from non-toxigenic strains.

Regarding amino acid requirements, *Cl. parabotulinum* type A closely resembles *Cl. sporogenes.* The unusually high demand for arginine, tyrosine and phenylalanine demonstrated by Shull *et al.* (1949) with *Cl. sporogenes* is shared by *Cl. parabotulinum* type A. The only difference observed with our organisms is an ability to utilize ornithine or lysine instead of arginine.

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(*Received* 27 *July* 1953)

ADDENDUM

After completion of this work 8 additional strains were examined for their vitamin requirements. Seven toxigenic strains showed the typical nutritional pattern—i.e. requirement for biotin, thiamine and *p*-AB. One strain which grew in absence of thiamine, when tested under conditions as described in the text, proved to be non-toxigenic.

We are indebted to Dr H. D. Michener of the Western Regional Research Laboratory, Albany, California, U.S.A., for 7 of the strains.

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Facultative Anaerobic Bacteria from the Sheep's Rumen

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SUMMARY: A study has been made of some 300 facultative anaerobic strains, isolated by Heald, Krogh, Mann, Appleby, Masson & Oxford (1953) from the rumen of a hay-fed sheep. The 120 streptococcal isolates all belonged to Lancefield's serological group D. 82 % had fermentation and other reactions similar to *Streptococcus bovis*; 6 % resembled *Str. faecalis* and the remainder (12 %) were unclassifiable by existing schemes. *Str. faecalis* could also be isolated from old hay fed to the sheep. Staphylococcal isolates were all coagulase-negative, and eleven out of twelve conformed to subgroup 2 in the classification of Shaw, Stitt & Cowan (1951). The coliform isolates were mostly of the true intestinal type. A few strains resembled *Aerobacter cloacae*, but *A. aerogenes* was not encountered. The large Gram-negative sarcina-like organism, previously detected in rumen contents by Baker, Nasr, Morrice & Bruce (1950) and by Moir & Masson (1952), has been isolated in pure culture and named *Sarcina bakeri*. It ferments only glucose.

In a recent paper (Heald *et al.* 1953) a method of counting viable saccharolytic bacteria from a sheep's rumen was described (hereinafter referred to as the 'bottle count') in which the basal medium consisted of rumen liquor itself, suitably clarified and sterilized after withdrawal from hay-fed sheep and supplemented by nothing more than a vitamin-free mixture of amino acids, a soluble fermentable carbohydrate and agar. In this way it was hoped that only bacteria genuinely active under rumen conditions would be counted. Several categories of soluble fermentable carbohydrates were tried, including hexoses, pentoses, glucosides, oligosaccharides, sugar alcohols and three polysaccharides (amylose, inulin and bacterial levan). By picking off colonies from the agar-shake cultures, some 300 isolates were eventually obtained in pure culture representing, for the most part, small cocci and Gram-negative rods present in numbers exceeding 100,000/g. wet rumen contents (see Table 2 in Heald *et al.* 1953). Since the methods used ensured that only the active carbohydrate fermenters would grow, these isolated bacteria were regarded as of actual or potential importance in rumen fermentations. This paper is concerned with the identification of these cultures, particular attention being paid to streptococci and other Gram-positive cocci, Gram-negative cocci and small Gram-negative rods. Gram-positive rods are not abundant in the rumen of mature hay-fed sheep, and the few isolates of Heald *et al.* which came into this category were ignored in this present survey.

It should be emphasized that the method of counting could be relied upon to demonstrate only the facultatively anaerobic microflora, and those microaerophiles (not in evidence in this study) which had no special carbon dioxide requirement. Evidence will be presented in another paper that true microaerophiles, from the calf's rumen, can be detected by our bottle-counting technique. A comparison between these rumen bacterial isolates and the microflora of the hay fed to the sheep has also been attempted.

METHODS

The cultures obtained by Heald *et al.* (all from sheep no. 879) were kept in cooked meat medium or in nutrient agar stabs, and before testing by standard bacteriological methods appropriate for the provisionally assigned genus were plated for purity on blood agar, the plates being incubated both aerobically and anaerobically. In the main the methods used for systematic study were taken from Mackie & McCartney (1950) and the *Manual of Methods for Pure Culture of Bacteria* (1948–9).

With streptococcal isolates, bacillary extracts prepared by the method of Fuller (1938) were in almost every instance tested for the precipitin reaction against Lancefield's Group D antiserum (Wellcome Brand, Burroughs Wellcome and Co.).

Other Gram-positive cocci were examined by the methods of Shaw *et al.* (1951).

RESULTS

Identification of streptococcal isolates. All truly streptococcal isolates from the rumen belonged to Lancefield's Group D, but fell into several categories with respect to fermentation reactions. The greater number seemed to be identical with the typical starch-fermenting streptococcus of the sheep's rumen, which has been described fully by MacPherson (1953). It is usually capsulated, gives rise to slight α-haemolysis on blood agar, ferments starch and (often) inulin, does not ferment mannitol, will grow at 45° but not at pH 9·6, nor in presence of 6·5% NaCl. In most respects it resembles *Str. bovis* (*Bergey's Manual*, 1948, p. 321) but apparently does not belong to the same serological type as this organism (MacPherson, 1953). It is quite distinct from *Str. faecalis* (Bergey, 1948, p. 325). The streptococcal isolates from our bottle counts all conformed to this (bovis) pattern when the following carbohydrates were used in the count; glucose (6 cultures isolated), sucrose (8), lactose (6), raffinose (7), salicin (19), inulin (1), amylose (6).

When mannitol, maltose or xylose was used in the bottle counts the streptococcal isolates were not all of this pattern (hereafter denoted 00++, the signs signifying no growth at pH 9·6, no fermentation of mannitol, but fermentation of starch and inulin respectively). Many of the mannitol bottle isolates were surprisingly of this pattern or of pattern +0++, i.e. they did not ferment mannitol after isolation, but representatives of patterns 0+++, 0++0, ++00 and +++0 (all fermenting mannitol) were also found. Of strains from the maltose bottles nearly 50% were of the pattern +0++, whilst both of the xylose bottle strains were of the pattern ++00, i.e. typical *Str. faecalis*. Those strains from mannitol and maltose which grew at pH 9·6 (see Shattock & Hirsch, 1947, for the usual significance of this behaviour) and yet were not the typical *Str. faecalis* by fermentation reactions were further tested for growth in 6·5% NaCl. Only one strain out of fourteen grew, and this was regarded as a starch-fermenting strain of *Str. faecalis*, so bringing the total number of *Str. faecalis* isolates to seven out of sixty-seven. The strains with pattern 0+++ were regarded as atypical representatives of the amylolytic rumen streptococcus with the added power of fermenting mannitol, so bringing the total number of these organisms to forty-five. Thus only fifteen streptococcal isolates (of patterns 0 + + 0 and + 0 + +) remained unclassifiable. These results are summarized in Table 1.

Table 1. *Classification of rumen streptococcal strains isolated from the bottle counts particularly with mannitol (MN) maltose (ML) and xylose (XL) as fermentable carbohydrates*

Four tests: growth at pH 9·6; fermentation of mannitol, starch and inulin: in that order.

Typical and atypical rumen amylolytic streptococcus patterns		*Str. faecalis* patterns		Unclassifiable patterns	
00 + +	0 + + +	+ + 00	+ + + 0	0 + + 0	+ 0 + +
27 (MN)	8 (MN)	4 (MN)	1 (MN)	2 (MN)	4 (MN)
10 (ML)		2 (XL)			9 (ML)
45 in all		7 in all		15 in all	
+ 53 from other sugars (see p. 143)					

Since the presence in the rumen of bacteria closely resembling *Str. faecalis*, although in relatively very small numbers, seemed of some interest, a fresh count was carried out with mannitol bottles in March 1953 on the rumen contents of hay-fed sheep no. 879 (the animal used by Heald *et al.*). Every colony developing at dilutions 1/10,000 and 1/70,000 was transferred to mannitol peptone water, and when fermentation took place, the culture was purified by plating and put through the usual series of fermentation and other tests. In this way five additional strains of *Str. faecalis* (pattern + + 00) were isolated, together with five similar strains of pattern + + 0 +, differing only in their power of fermenting inulin. All ten strains grew at 45° and all fermented in addition (with production of acid and no gas) lactose, salicin, raffinose, sorbitol and trehalose. None liquefied gelatin. Five out of ten strains survived heating at 60° for 30 min. All clotted and decolorized litmus milk. Save for the fermentation of raffinose, a property shared by all the so-called *Str. faecalis* strains isolated in this study, this behaviour is in harmony with that of *Str. faecalis* from human sources. In point of fact an authentic strain of *Str. faecalis* (NCTC 775) also fermented raffinose under our conditions.

The Gram-positive cocci of the hay fed to sheep no. 879. A study of the facultative anaerobic microflora of the fodder was made to find whether the rumen *Str. faecalis* was in reality merely a 'passenger' derived from the hay, which being kept in quantity before use in an open box might have collected bacteria from the dust in the air. The fairly finely divided hay (10 g. of an average sample) was shaken with sterile Ringer's solution (25 ml.) first by hand for 10 min., and finally on the machine for 45 min. The fluid part was decanted into a sterile centrifuge tube, and the deposit obtained after long centrifuging at 2000 r.p.m., was re-suspended in sterile Ringer's solution (5 ml.). The bottle-counting procedure (Heald *et al.* 1953) was then carried out on this liquid, glucose being used as fermentable carbohydrate. Contrary to the results of Heald *et al.* (1953) with a different sample of hay, an almost exclusively streptococcal flora developed in this instance. Twenty colonies were picked at random and were purified by repeated plating on glucose yeast-extract peptone agar. After purification, all isolated strains behaved exactly as *Str. faecalis*, i.e. grew at pH 9·6, and in 6·5% NaCl, fermented mannitol and trehalose, but not starch. They also clotted and decolorized litmus milk. In further resemblance to the rumen *Str. faecalis*, all strains fermented raffinose. Some strains also fermented inulin (see above).

Just after this experiment the hay fed to the sheep was changed, the fodder box being charged with newly purchased hay. An attempt to study the streptococcal flora of this new hay failed however, the only colonies developing in the bottle counts being Gram-positive rods which overgrew everything else when colonies in the depths of the agar were plated out. It has not therefore been possible to find whether the *Str. faecalis* in the old hay was really derived from the hay itself. or from the dust of the animal house in which the sheep was kept.

Other Gram-positive cocci from the rumen. A few of the isolates reported as streptococci by Heald *et al.* (1953, see Table 2 in that paper) were found upon re-examination to be staphylococci or micrococci. They yielded large catalase-positive non-haemolytic colonies on blood agar and showed bacteria in tetrads and groups, as well as in short chains, in stained preparations. For example, four out of fifty isolates from the mannitol bottles, six out of twelve from the glucose bottles, five out of twelve from the raffinose bottles and two out of twenty-one from the salicin bottles conformed to this description. They were re-examined at the same time as other isolates definitely reported by Heald *et al.* as 'other Gram-positive cocci'. Twelve representative strains were examined by the methods of Shaw *et al.* (1951). The cultural characters as found are listed in Table 2, and it will be seen that with one exception, viz. the culture from the sucrose bottle count, they are all VP-positive and conform in most other respects to subgroup 2 of the classification of Shaw *et al.* (see their table 1, p. 1017). In view of the very weak fermentation reactions of the solitary 'sucrose' strain it may perhaps best find a place in their subgroup 5. It is remarkable, however, that it should hydrolyse starch. This property may indicate its role in the rumen.

Coliform bacteria. Although some 150 isolates of coliform bacteria were obtained, mostly from the mannitol and xylose bottle counts, a complete study of all these strains was not made since their relative numbers in the rumen, when the sheep is on a hay diet, are quite small, being merely of the order of 10^4–10^5/g. wet rumen contents. Six strains from mannitol, six from xylose, three from rhamnose and three from sorbitol bottles were chosen at random and were all found to be typical intestinal *Escherichia coli* by IMViC reactions and gas production from lactose at 44°. Capsulation was not found in any of the 150 strains (observation by Miss Elizabeth Mackay using the indian ink technique), and it is highly probable that very few indeed, if any, corresponded to the typical *Aerobacter aerogenes*.

Table 2. *Cultural characters of rumen micrococci*

	Sugar used in bottle count for isolation				
	Glucose	Raffinose	Xylose	Sucrose	Salicin
No. of strains isolated ...	5	3	1	1	2
	Properties of strains				
Opt. temperature: 22°	0	0	0	0	0
30–37°	5	3	1	1	2
Pigment on potato: none	5	3	1	0	0
lemon-yellow	0	0	0	1	0
golden-yellow	0	0	0	0	2
NH_3-salt utilization	4	0	1	1	0
MR positive	5	0	1	0	2
VP positive	5	3	1	0	2
H_2S produced	0	0	0	0	0
Nitrate reduced	5	3	1	0	0
Methylene blue reduced	5	3	1	1	2
Litmus milk: no change	0	3	0	1	0
acid	0	0	1	0	2
acid clot	5	0	0	0	0
digestion	5	0	0	0	0
Gelatin liquefied	5	0	0	1	2
Serum digested	0	0	0	0	0
Coagulase positive	0	0	0	0	0
Acid from:* glucose	5	3	1	1 (weak)	2
xylose	0	0	0	0	0
galactose	5	3	1	0	2
lactose	5	3	1	0	2
sucrose	5	3	1	0	2
maltose	5	3	1	1 (weak)	2
raffinose	1	0	0	0	0
starch	0	0	0	1	0
dextrin	5	3 (1 weak)	1	0	2
glycerol	5	3	0	0	1
mannitol	0	3	1 (weak)	0	0
dulcitol	0	0	0	0	0
salicin	0	0	0	0	0
trehalose	0	3	1	0	2
Urea hydrolysed	5	3	1	0	2

* No strain fermented arabinose, rhamnose, inositol, inulin, adonitol or aesculin.

'Other Gram-negative rods' mentioned by Heald *et al.* (1953), which on isolation appeared not to ferment lactose on MacConkey agar, were later found to ferment this sugar slowly in a liquid medium. Five strains were examined in detail (three from raffinose and two from mannitol bottle counts) and all proved to be motile, indole-negative, methyl red-negative, VP-positive, citrate-positive, gelatin-liquefying non-capsulated, small Gram-negative rods, that is conforming in all respects to *Aer. cloacae*, rather than to *Aer. aerogenes*.

Gram-negative cocci. Heald *et al.* (1953) isolated several pure cultures of Gram-negative cocci from the mannitol and xylose bottle counts. Nine isolates from the xylose bottles and one from a mannitol bottle were examined in detail and proved to be identical in all respects. This organism, which grows well in ordinary nutrient media, appears to be the well-known rumen sarcina, photomicrographs of which have already been published by Baker *et al.* (1950, figs. 43–45) and Moir & Masson (1952, organism no. 26, figs. 10, 16 and 17). Although our isolates were neither iodophile nor capsulated in pure culture on artificial media, nevertheless the large size, sarcina-like arrangement and persistent Gram-negative nature of the cocci render an identification with Baker's rumen organism (which he did not isolate) highly probable. In spite of its Gram-negative character, we propose to place this organism in the genus *Sarcina* and to name it *S. bakeri*, after the late F. Baker, whose elegant photographic techniques have brought to light many clues for the identification of true rumen bacteria of medium size.

Sarcina bakeri n.sp. Spherical or slightly oval cocci $4\mu.$ in diameter or larger when first isolated; dwindling to $1–2\mu.$ after many subcultures on artificial media; occurring in pairs (with long axes parallel), tetracocci or closely packed larger groups. Non-motile, no endospores. Capsules not observed in pure culture, although said to be present in the rumen of a sheep receiving starch in the ration (Moir & Masson, 1952). Gram-negative always, even when young. Colonies on nutrient agar raised, greyish white, translucent with entire edge, 1·3–2 mm. diameter. Grows readily in nutrient broth, forming smooth uniform turbidity with abundant deposit and thin, easily disintegrated surface pellicle. Facultative anaerobe. Grows vigorously at 30 and 39°, but only poorly at 20°. Ferments glucose only, with production of acid but no gas. Grows well in non-carbohydrate media such as peptone water. Does not liquefy gelatin, nor change litmus milk. Catalase-positive. Voges-Proskauer, methyl red, indole and H_2S tests all negative. Does not reduce nitrate. Isolated at Bucksburn, Aberdeenshire, from the rumen of a hay-fed sheep in 1952. The products from a glucose fermentation by *S. bakeri* are now under examination.

DISCUSSION

It has recently been recognized (Hungate, Dougherty, Bryant & Cello, 1952) that saccharolytic bacteria, particularly streptococci, even if at one time present in relatively small numbers in the rumen, may nevertheless quickly become of much greater importance later on when the soluble carbohydrate content of the fodder is suddenly increased by drastically altering the ration fed to the animal. The sheep we have worked with was kept on the simplest possible ration (hay) containing very little starch or soluble sugar. Hence in all probability the saccharolytic rumen micro-flora found is numerically at the basic minimum, and it would be unwise to infer that the intestinal coliform bacteria, which seemingly constitute a very small part of it, are not 'true' rumen organisms, and that they could never under any circumstances play an important role in the rumen. It is significant that *Aer. aerogenes*, the common saprophyte found on plants and grains, was not found in the rumen. Furthermore, the short-term fermentation studies of Heald (1952) with xylose and rumen liquor, showed that coliform bacteria of the intestinal type are in a very active state in the rumen since they grew very rapidly indeed and soon predominated when the pentose was supplied. Our bottle counts with xylose also yielded numerous coliform bacteria of the intestinal type, partly no doubt because the predominant rumen streptococcus does not ferment this sugar (MacPherson, 1953).

There seems also to be a great diversity of fermentative pattern among the rumen streptococci, some of which are unclassifiable at the moment. It would be of interest to know whether this diversity is found in lambs beginning to ruminate, or whether it is a function of the age of the sheep.

The isolation in pure culture of a large Gram-negative sarcina-like organism, which on morphological grounds alone may almost certainly be regarded as a true rumen organism since it has so frequently been seen in rumen contents, is, we think, good evidence of the essential soundness of our bottle counting and isolation technique. It is at first sight surprising that this and many other isolates failed to ferment, in peptone water, the carbohydrates added to the agar in the bottles from which they were isolated. In the case of *S. bakeri*, this might have been due to the ability of the organism to grow on amino acids alone. In other instances, e.g. streptococci from mannitol bottles, it would seem that other bacterial colonies (coliforms?), developing in the same bottle, provided nutriment for the non-mannitol-fermenting streptococci. Such symbiotic relationships are probably very common in the rumen, and some of them may be brought to light only if, as in our technique, an agar containing the minimum amount of nutriment is used for the isolation of the rumen bacteria.

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(*Received 5 August* 1953)

HART, P. D'A. & REES, R. J. W. (1954). *J. gen. Microbiol.* 10, 150–159.

Changes in Morphology of an Avirulent Strain of *Mycobacterium tuberculosis* under the influence of Non-ionic Surface-active Agents

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SUMMARY: When the avirulent strain of *Mycobacterium tuberculosis* H37Ra was repeatedly subcultured in a liquid medium containing low concentrations of 'Triton A20' or other chemically similar non-ionic surface-active polyoxyethylene ethers, the morphology changed from the amorphous growth pattern characteristic of avirulent tubercle bacilli to the formation of microscopic 'cords' similar to those shown by virulent strains such as H37Rv. Once developed, the cord-forming property of H37Ra persisted when cultures were transferred to liquid medium devoid of surface-active agent, even when such transfer was made after passage through mice; after further subcultures, however, a tendency to reversion was apparent. No difference in virulence was detected between cord-forming H37Ra and the unmodified strain when tested in mice.

Previous work had indicated that a lower member of the polyoxyethylene ether series, the dimer 'D4', inhibited amorphous-growing avirulent tubercle bacilli, including H37Ra, but not the growth of naturally cord-forming strains, exemplified by the virulent H37Rv and attenuated strains derived from it. The growth of cord-forming avirulent organisms, developed from H37Ra in the present experiments, was also found to be relatively unaffected by D4.

During an investigation of the properties of an attenuated strain of *Mycobacterium tuberculosis* (VAL, see Rees & Hart, 1952), derived from the virulent human variant H37Rv, we noted that virulence could be partly restored by repeated subculture in a medium containing the commercial non-ionic surface-active agent 'Triton A20' (Hart & Rees, unpublished). Tests were therefore made of the behaviour of some standard avirulent strains of *M. tuberculosis* after similar treatment. The morphology was strikingly changed in one such strain, although no significant virulence was induced. It is a characteristic of virulent tubercle bacilli to form microscopic 'cords' when grown in certain liquid media, whereas avirulent strains grow amorphously. The change in morphology here to be described was away from an amorphous, and towards a cord, pattern of growth.

MATERIALS AND METHODS

Surface-active agents. 'Triton A20' was made by Rohm and Haas Co., Philadelphia, and supplied by Charles Lennig and Co., Ltd., London; it is stated to be a 25% solution of 'Triton WR1339', a polymeric *p-iso*octyl polyoxyethylene phenol (see Rees & Hart, 1952; Friedman & Byers, 1953). 'Tween 80', a polyoxyethylene derivative of sorbitan mono-oleate, was made by Atlas Powder Co., Wilmington, Delaware. The other agents were non-ionic surface-active polyoxyethylene ethers which had been synthesized by our colleagues Drs J. W. Cornforth and J. A. Stock in the course of studies following the observation that certain products of this type (including 'Triton A20') suppressed the development of experimental tuberculosis in mice and guinea-pigs (Cornforth, Hart, Rees & Stock, 1951; Hart, Long & Rees, 1952; Rees, 1953). The general formula of this series has been given elsewhere (see Rees & Hart, 1952). The products used here were the dimer D4 (the ether of 2, 2′-dihydroxy-5, 5′-di-*tert*-octyldiphenylmethane, with an average of twenty ethylene oxide units per phenolic nucleus); the trimer D9a (also with twenty ethylene oxide units per nucleus); and the polymeric mixture D27 (twelve ethylene oxide units per nucleus). 'Triton A20' 50 % (12·5 % effective agent), or the other products at 12·5 %, were autoclaved and, before use, diluted to 1·0 %. (All concentrations of 'Triton A20' given hereafter refer to the effective agent.)

Cultures. The strain of *M. tuberculosis* mainly used was H37Ra, the avirulent variant of the human strain H37; this, as well as the virulent variant H37Rv and four additional avirulent human strains, R1Ra, JH6Ra, JH16Ra and H4Ra, were obtained from Dr W. Steenken, Jr., Trudeau Laboratory, New York (see Report, 1946; Steenken & Gardner, 1946*a*, *b*; Yegian & Kurung, 1952).

Media and tests. The avirulent strains of *M. tuberculosis* were maintained on Herrold's (1931) egg-yolk agar medium, containing 5 % glycerol, and the virulent strain H37Rv on the surface of Proskauer & Beck's defined medium containing asparagine (see Baldwin, Petroff & Gardner, 1927). Well-grown cultures were ground in Griffith tubes to give aqueous suspensions equal in opacity (by eye) to a standard containing 0·2 mg. (wet-weight)/ml. The liquid medium used for the repeated subculture and other tests had the following basal constituents: Difco Casamino acids, 2·5 g.; asparagine, 0·3 g.; anhydrous Na_2HPO_4, 2·5 g.; KH_2PO_4, 1·0 g.; sodium citrate, 1·5 g.; $MgSO_4$, $7H_2O$, 0·6 g.; glycerol, 25 ml.; distilled water to 1000 ml. This basal medium was distributed in 5 ml. quantities in 25 mm. diameter test tubes and autoclaved; and to each tube 0·5 ml. 5 % albumin (bovine plasma fraction V) sterilized by filtration, and a surface-active agent to give the required concentration, were added, the product being referred to hereafter as 'the liquid medium'. The inoculum, 0·2 ml. of a standard suspension (0·04 mg. wet-weight bacilli) or 0·1–0·2 ml. of a culture grown in similar liquid medium, was then added, and the tubes incubated at 37° for 2–3 weeks. Alternatively, for *in vivo* experiments, the basal medium was distributed in 50 ml. quantities in 250 ml. conical flasks. The additions were then 5 ml. 5 % albumin, a surface-active agent, and the inoculum (1 ml.) from a culture in a tube of similar medium; incubation was for 7–10 days (the flasks being shaken by circular motion twice daily to promote dispersion when a concentration of 0·001 % 'Triton A20' was used).

Examination of cultures. The degree and character (granular or dispersed) of submerged growth in the liquid medium were determined by naked-eye, or with a hand-lens, after gentle shaking. The morphology was assessed by the microscopic examination, under 2 mm. oil-immersion lens, of films prepared by placing loopfuls of the cultures on slides, fixing, and staining by the Ziehl-Neelsen method.

Inoculation of mice. Cultures in the flasks (see above) were centrifuged, and the bacterial deposits re-suspended in 'Tween 80' + albumin + saline solution to give 1 mg. (wet-weight)/ml. (measured by a Spekker photo-electric absorptiometer). The bacillary suspensions, diluted in Tween + albumin + saline to contain the required doses in 0·5 ml., were injected into the tail veins of female mice (18–20 g.) of the albino P strain. In one experiment the suspensions were prepared to standard visual opacity by grinding from egg-yolk agar cultures, and 0·5 ml. (0·1 mg. wet-weight bacilli) used to inject by the same route.

RESULTS

Effect of repeated subculture in the presence of 'Triton A20' on the morphology of the avirulent strain H37Ra

Samples of suspensions prepared from egg-yolk agar cultures of H37Ra were inoculated into tubes of the liquid medium containing 'Triton A20' 0·001 or 0·002 %. After incubation, samples from these tubes were seeded into fresh tubes of medium containing the same concentrations of agent, and the process of transfer and culture repeated. These concentrations were chosen for three reasons: they had been effective in enhancing virulence of the VAL strain; microscopic 'cords' were more evident when VAL or H37Rv was grown in these than in higher concentrations, such as 0·05 %; and in any case only low concentrations of 'Triton A20' permit the growth of avirulent strains of *M. tuberculosis* (Rees & Hart, 1952). Growth was slow and sparse but appeared to be continuous; it was submerged and was granular or partly dispersed. In a typical experiment, microscopic examination of the first culture in the medium containing 0·001 % agent showed the bacilli arranged in characteristic amorphous clumps (Pl. 1, fig. 1*a*), but a change was already evident after the second exposure during growth. The third serial culture showed semi-amorphous clumps of bacilli with some cord formation (Pl. 1, fig. 1*b*), while the tenth showed well-defined cords without any amorphous collections (Pl. 1, fig. 1*c*); this appearance was similar to that displayed by the virulent strain H37Rv after one culture in the same medium (Pl. 1, fig. 2). Cord formation developed much less in the series grown in the presence of 'Triton A20' 0·002 %.

The degree and rate of change in morphology of H37Ra varied considerably, and after cord formation had been induced, further transfers did not improve and might even reduce it. At the peak the cords were usually less tightly packed, less serpentine, and more branched, than those of H37Rv. Throughout the series the organisms remained strongly acid-fast.

To see whether prolonged growth would produce cords as well as repeated subculture, a first culture of H37Ra in 'Triton A20' 0·001 % was incubated for 7 weeks. Examination at 2 weeks showed the expected amorphous growth pattern; and there was no appreciable change at 7 weeks.

Stability of the microscopic cord pattern of growth of H37Ra

Stability of the cord-forming property was examined in two ways, using in each case tubes taken from a series of subcultures (ranging from the 6th to the 14th) in the liquid medium containing 'Triton A20' 0·001 % after the stage of well-defined cord formation had been reached. In the first method samples from such tubes were sown on to egg-yolk agar, and suspensions prepared from the resulting cultures were inoculated (0·04 mg.) into tubes of the usual medium containing 'Triton A20' 0·001, 0·002 or 0·05 %, as well as in the same medium without any agent (i.e. basal constituents plus albumin only). In the second method a sample from one such tube (11th in the series) was seeded (1 ml.) into a flask of the liquid medium containing 'Triton A20' 0·001 %, or 0·2 and 0·5 mg. (wet-weight bacilli) of the resulting culture inoculated into two groups of six mice. One of the animals (0·2 mg. group) died 32 days later with several large foci in the lungs; the other eleven were apparently well at 39 days, when they were killed, and pin-point pulmonary foci were found in the lungs of eight and more definite though limited lesions in those of three (0·2 mg. group). Cultures on to egg-yolk agar were made from the pulmonary lesions of the mouse that died and of one of those killed, after grinding with sand and treating the suspensions with acid; the cultures were tested precisely as in the first method.

The tests made with the egg-yolk agar cultures obtained by both these methods still showed a microscopic cord pattern of growth, not only in the presence of 'Triton A20' 0·001 or 0·002 %, but also in the liquid medium without any agent. The appearances after passage through mice to egg-yolk agar, and thence to the liquid medium devoid of the agent, are shown in Pl. 2, fig. 3*a*, and in the presence of the agent 0·001 % in Pl. 2, fig. 3*b*. Some, though slow, growth of both sets of cultures also took place in the presence of 'Triton A20' 0·05 %; this growth was dispersed but microscopically amorphous. Egg-yolk agar cultures of unmodified H37Ra and of H37Rv, inoculated into similar tubes for comparison, showed an amorphous growth pattern and well-marked cord formation, respectively, in the presence of 'Triton A20' 0·001 or 0·002 %. In 0·05 % of the agent the growth of the unmodified H37Ra was inhibited (see above), while H37Rv showed profuse short cords, as was to be expected.

When egg-yolk agar cultures of cord-forming H37Ra were subcultured once again on egg-yolk agar before being inoculated into the liquid medium devoid of surface-active agent, partial reversion to the amorphous growth pattern was observed.

Effect of the changed morphology on the inhibition of H37Ra *by the surface-active polyoxyethylene ether* D4

We have previously shown that one member of the series of surface-active polyoxyethylene ethers made in this Institute (the dimer D4) inhibited the growth of avirulent strains of *M. tuberculosis*, whereas virulent, as well as attenuated, strains grew freely in its presence; a factor common to the strains naturally insusceptible, but absent from those inhibited, is the ability to produce some degree of microscopic cord pattern during growth (Rees & Hart, 1952). We set out now to determine whether the induced production of cords by H37Ra was associated with a loss of susceptibility to D4. The observation, recorded above, that subcultures on egg-yolk agar of cord-forming organisms derived from H37Ra would grow in moderate concentrations (0·05 %) of 'Triton A20' (which mixture contains a significant amount of the dimer), whereas the original amorphous-growing H37Ra was inhibited by such concentrations, had given a hint of a change in susceptibility. Further use was made of these egg-yolk agar cultures (obtained from subcultures in the series in the medium containing 'Triton A20' 0·001 %) during the performance of the stability tests already described. Suspensions prepared from these cultures (whose cord-forming property persisted when grown in liquid medium containing 'Triton A20' at low concentrations or in its absence, see above), and also from egg-yolk agar cultures of the unmodified H37Ra, were tested simultaneously for growth inhibition by D4, using the usual liquid medium and comparable inocula (0·04 mg.). The inhibitory end-point after 2 weeks' incubation was found to be >0·1 % for the cord-forming strains in contrast to 0·006 % for the unmodified strain, though the growth of the former was sometimes first evident only during the second week. Microscopically, the cord-forming strain grew amorphously in D4 0·05 %, as in that concentration of 'Triton A20', and the property of cord formation was much less striking in low concentrations of the dimer than of the latter agent.

Effect of the concentration of 'Triton A20' on cord formation

The optimal concentration of 'Triton A20' for cord formation by H37Ra after exposure during repeated subculture appeared to be about 0·001 %. Certainly 0·002 % was less effective. Lower concentrations than 0·001 % were not tested, but eight subcultures in liquid medium without any agent (i.e. containing albumin 0·5 % alone) was unsuccessful in producing cords. As already stated, unmodified H37Ra fails to grow in 'Triton A20' 0·05 %, while cord-forming strains, after transfer to egg-yolk agar, grow amorphously in this concentration. It was found, moreover, that such strains, when transferred from liquid medium containing 'Triton A20' 0·001 % (in which they showed well-marked cords) directly into similar medium containing 0·05 %, also grew amorphously; but when the latter culture was inoculated (0·1 ml.) back into the medium but without agent (giving a final concentration again of 0·001 %), cords reappeared.

Effect of repeated subculture in other surface-active agents

The strain H37Ra was subcultured repeatedly in liquid medium containing low concentrations of several members of our series of polyoxyethylene ethers. Cords developed in the presence of 0·001 % D4 or D9a, and 0·002 % D27, at the same rate as in 'Triton A20'; the macroscopic character of the growth was similar. It should be mentioned also that on two occasions cultures of H37Ra which had been transferred a number of times from and to the liquid medium containing 'Tween 80' 0·05% (the growth being amorphous, as would be expected), and which were then transferred to egg-yolk agar and finally to medium containing 'Triton A20', showed well-marked cord formation. These observations with 'Tween 80' were not reproducible at will.

Behaviour of other avirulent strains of M. tuberculosis

The four avirulent strains R1Ra, JH6Ra, JH16Ra and H4Ra were repeatedly subcultured in the presence of 'Triton A20' by the method used for H37Ra. No clear evidence of cord formation was noted in any strain after at least seven exposures to the agent 0·001 or 0·002%. The organisms of the H4Ra strain were strongly acid-fast, but those of the other three were largely acid-susceptible and remained so. In the case of H4Ra, although unsuccessful by serial culture, we have twice observed definite cord formation in the liquid medium containing this type of surface-active agent. The reproducibility of this phenomenon is being investigated.

Virulence tests with the cord-forming strains

Tubes from a series of subcultures (4th, 7th and 11th) of H37Ra in the liquid medium containing 'Triton A20' 0·001%, after the stage of cord formation had been reached, were used to inoculate flasks of the same medium and 0·001 or 0·002% of this agent; after growth, 0·2 and 0·5 mg. (wet-weight bacilli) of the cultures were used to infect mice. Only pin-point pulmonary lesions were seen when the animals were killed 40 days later, except in one group (11th subculture) where more definite lesions were present in some animals and in the only one that had died of the disease (p. 153). Even though egg-yolk agar cultures made from these apparently more significant lesions were still capable of forming cords in liquid medium, they produced no deaths and only pin-point lesions when used (0·1 mg.) to infect a fresh group of mice.

Cytochemical test. Egg-yolk agar cultures of cord-forming and of unmodified H37Ra were used for the cytochemical test (Dubos & Middlebrook, 1948). In neither case did the organisms take up the neutral red.

Character of H37Ra *on Proskauer and Beck's medium*

Although we have, in general, maintained H37Ra on egg-yolk agar, we are aware that the Trudeau Laboratory, which supplied our transplant, carries avirulent as well as virulent strains of tubercle bacilli on the surface of Proskauer and Beck's fluid medium, as a suitable medium for keeping these strains stable (Report, 1946). No increase of virulence in H37Ra has been noted after the use of this method for many years (Dr W. Steenken, Jr., personal communication). We found that H37Ra, taken from egg-yolk agar and grown on Proskauer and Beck's medium, did not produce cords spontaneously in the pellicle, and was no more likely to produce cords on a first subculture from Proskauer and Beck's medium in our liquid medium containing 'Triton A20' 0·001% than when a first subculture therein was made from egg-yolk agar.

DISCUSSION

Although the arrangement of the bacilli in amorphous clumps appears to constitute the normal *in vitro* microscopic growth pattern of the standard avirulent strains used in the present investigation (Yegian & Kurung, 1952), 'spontaneous' changes in morphology towards the formation of 'cords' have been occasionally noted previously. Yegian & Kurung themselves mention one culture of H37Ra which, after 3 years' maintenance in their laboratory in Tween + albumin medium, exhibited definite cord formation microscopically when cultivated in their test medium. The formation of cords has been regularly observed when H37Ra is grown in oleic acid + albumin medium, or in Gey's balanced salt solution, to which chick embryo material has been added (Bloch, 1948; Friedmann & Russell, 1953). The morphology reverts to the usual unoriented pattern of growth when the strain is returned to ordinary media devoid of this material.

The present findings with H37Ra differ in three respects from those in media containing embryonic tissue. The medium is more clearly defined in composition, its active component appearing to be a synthetic non-ionic surface-active agent. Cords are not produced on first exposure but only after several subcultures in the effective medium. Once obtained, this property persists after transfer to medium from which the agent is absent, whether the transfer be direct or after passage through mice; after further subcultures, however, there is a tendency to reversion.

The methods reported here for inducing cord formation are empirical. That successful for H37Ra is defined (though with inconstancy in the time of onset and quality of cords), but the few observations of cords in the strain H4Ra have not yet been systematized. Several non-ionic surface-active agents promote the change, and, if 'Tween 80' be included, it is not attributable to any one chemical series. The conditions of cultivation for successful induction in H37Ra—low concentrations of agent—are also optimal for manifesting the cords when their formation has been achieved. Yet the modification of morphology is not just due to the coincident presence of the surface-active agent, since it is still evident after return of the strain to the ordinary egg medium and subsequent subculture into the liquid medium also devoid of the agent.

The loss of the selective inhibitory effect of the dimer D4 on the growth of H37Ra, when the latter comes to form cords in 'Triton A20', is in keeping with our previous experience that the growth of any strain of tubercle bacillus which shows some degree of cord formation is relatively unaffected by D4 (Rees & Hart, 1952). We had, however, previously found it easy to produce avirulent bacilli resistant to D4 or to 'Triton A20' (which contains D4) *without* loss of the typical amorphous growth pattern, by subcultivating H37Ra in increasing concentrations of these agents. The question therefore arises whether the loss of susceptibility to D4 in the present experiments is due to 'resistance' rather than to cord formation. This seems improbable because the exposures of H37Ra during growth were to a constant low concentration (0·001 or 0·002 %), and not to increasing strengths, of 'Triton A20'. Moreover, the growth of a culture of H37Ra in which cord formation had been induced in the presence of 'Tween 80', whose basic structure is unrelated chemically to that of D4, was also found to be uninhibited by the latter.

Little information can be offered on whether the mechanism responsible for cord formation in an avirulent strain of *M. tuberculosis* is a selection of mutants or a direct 'adaptation'. An attractive hypothesis, suggested by the considerations in the previous paragraph, is that D4 or 'Triton A20', by inhibiting the growth of amorphous avirulent organisms and permitting the growth of cord-forming mutants, favours selection of the latter during serial subculture. This hypothesis might account for cord formation by H37Ra in these two surface-active agents, though the facts that their effective concentrations are subinhibitory and that exposure of H37Ra to increasing concentrations is more likely to give 'resistant' amorphous organisms than 'cord-formers' (see above) make it unlikely. The hypothesis could not, however, explain the phenomenon when it occurs in the presence of the trimer D9a, which has no growth-inhibitory action on avirulent or virulent bacilli (Rees & Hart, 1952). Although this particular form of selection of mutants appears, therefore, to be excluded, mutation itself is not. In this connexion, the observation that prolonged incubation of a first culture of H37Ra in the presence of a concentration of surface-active agent which is effective during repeated subculture fails to induce significant cord formation has some interest.

A broad correlation has been suggested between the morphology of mammalian tubercle bacilli and their virulence, namely, that when cultivated under specific conditions virulent strains form cords, avirulent strains grow amorphously, while attenuated strains present an intermediate pattern (Middlebrook, Dubos & Pierce, 1947; Middlebrook, 1950). A more restricted definition of the relationship is given by Yegian & Kurung (1952): 'while all cord-formers are not necessarily virulent, all virulent strains are cord-formers'. Nevertheless, the fact that cord formation can be induced in originally amorphous-growing standard avirulent strains makes it important to know whether virulence has appeared. Yegian & Kurung observed gross lesions in the liver and spleen of a guinea-pig inoculated with their cord-forming culture of H37Ra (H37Ra(x)), whereas a typical amorphous-growing culture gave no such lesions. Bloch found that when mice were given comparable doses of H37Ra grown in medium containing chick embryo extract and in medium without this addition, neither group showed definite evidence of virulent infection by clinical deterioration or at autopsy. We have been unable to distinguish consistently the pathogenicity of cord-forming H37Ra from that of unmodified H37Ra in mice after heavy dosage, either on the basis of mortality or of gross pathology, even though cord-forming organisms could be recovered from the pulmonary lesions. This experience indicates that the correlation between virulence (as ordinarily measured) and cord formation is by no means close. Similar evidence is provided by strains with degraded virulence derived from H37Rv, which may show cords indistinguishable from those of the latter. On the other hand, these findings do not controvert the more restricted definition of the relationship between morphology and virulence referred to above.

In the cytochemical reaction, it is stated that virulent tubercle bacilli which form serpentine cords on growth, such as H37Rv, bind neutral red in alkaline medium, whereas avirulent strains such as H37Ra do not (Dubos & Middlebrook, 1948). Our finding that cord-forming H37Ra behaved in the test like the unmodified strain may have little significance, since in our hands the differences in coloration between H37Ra and H37Rv have been only slight (cf. Richmond & Cummings, 1950).

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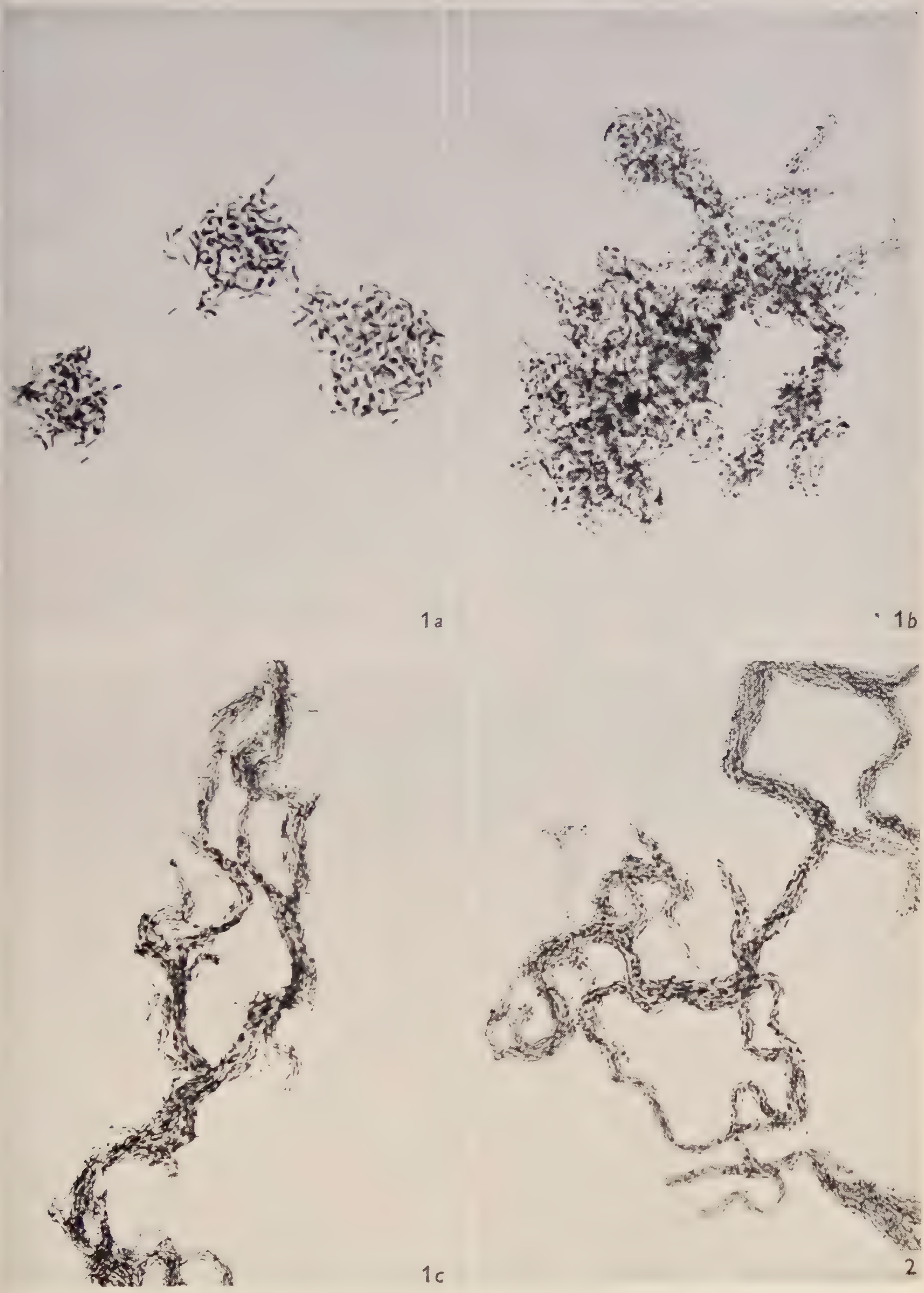
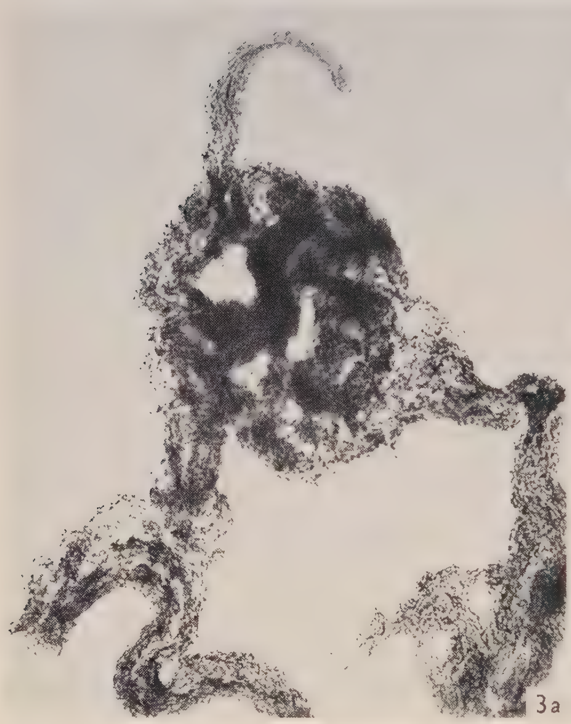


P. D'Arcy Hart & R. J. W. Rees—Morphology of avirulent tubercle bacilli. Plate 1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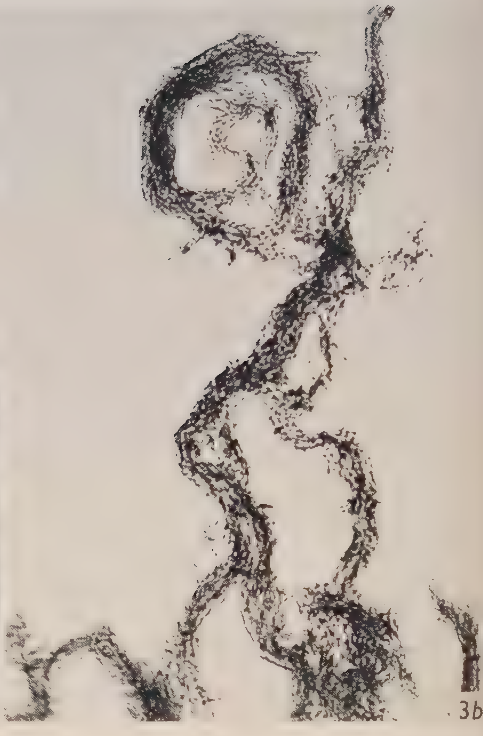
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P. D'Arcy Hart & R. J. W. Rees—Morphology of avirulent tubercle bacilli. Plate 2

EXPLANATION OF PLATES

Plate 1

Fig. 1. *a–c*: *Mycobacterium tuberculosis*, avirulent strain H37Ra, cultured repeatedly in the test liquid medium containing 'Triton A20' 0·001 %. Ziehl-Neelsen stain. *a*, first serial culture, after 2 weeks' growth. Shows amorphous collections of bacilli. × 900. *b*, third serial culture, after 2 weeks' growth. Shows semi-amorphous collections of bacilli, with commencing formation of cords. × 800. *c*, ninth serial culture, after 2 weeks' growth. The bacilli are arranged in fairly well-developed cords. × 800.

Fig. 2. *Mycobacterium tuberculosis*, virulent strain H37Rv, after 2 weeks' growth in the test liquid medium containing 'Triton A20' 0·001 %. Shows well-developed 'tight' serpentine cords (compare with Fig. 1*c*). Ziehl-Neelsen stain. × 800.

Plate 2

Fig. 3. *a*, *b*: Cultures of organisms recovered, via egg-yolk agar, from the lungs of mice infected with cord-forming H37Ra. Shows persistence of cord formation. Ziehl-Neelsen stain. *a*, grown in the test liquid medium without surface-active agent. × 650. *b*, grown in the test liquid medium containing 'Triton A20' 0·001 %. × 800.

(*Received 7 August* 1953)

BAWDEN, F. C. & KASSANIS, B. (1954). *J. gen. Microbiol.* **10**, 160–173.

Some Effects of Thiouracil on Virus-infected Plants

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SUMMARY: Submerging leaves in water soon after they are inoculated with viruses can prevent infection, and for studying factors that affect the rate of virus multiplication leaves should not be placed in solutions until a day after inoculation.

The rate at which viruses multiply in tobacco leaves is decreased by spraying with solutions of thiouracil, but less so than by floating leaves in the solutions. The physiological state of floated leaves affects the extent to which thiouracil impedes virus multiplication; least virus is produced in the presence of thiouracil when the condition of leaves otherwise most favours virus formation. Multiplication of virus can be checked at any time by thiouracil, but is most affected when leaves contain little virus; multiplication is resumed when thiouracil is removed. Thiouracil impedes the multiplication in tobacco of all viruses tested, but not of a tobacco necrosis virus in French bean or broad bean mottle virus in *Vicia faba*.

When mixed with inocula, thiouracil can prevent infection from occurring, both in tobacco and French bean. It also affects the growth and appearance of both plants. These effects, unlike the impedance of virus multiplication in tobacco, are not counteracted by an excess of uracil. Tobacco leaves in which tobacco mosaic virus is multiplying develop necrotic spots and rings when treated with thiouracil, and local lesions can be made evident by spraying inoculated leaves. Necrotic lesions also occur on *V. faba* infected with broad-bean mottle virus and treated with thiouracil.

Commoner & Mercer (1951, 1952) found that thiouracil inhibits the production of tobacco mosaic virus in tobacco leaves and that its inhibitory action is counteracted by an excess of uracil. In their experiments, detached tobacco leaves were rubbed over their whole upper surfaces with the virus, and kept for 24 hr. at 24° under a bell jar in continuous light of 120 f.c. Disks 12 mm. in diameter were then cut from the leaf blades and floated in a nutrient solution with various concentrations of thiouracil and uracil. The disks were kept at 24° and in continuous light of 120 f.c. until they were macerated for assays of their virus contents. We have not used these exact conditions, but we have repeatedly confirmed the observation that thiouracil can inhibit the production of tobacco mosaic virus and that uracil neutralizes this effect of thiouracil. By working under different and varying conditions, we have found that the extent to which thiouracil inhibits virus production varies with the physiological condition of the leaves. Perhaps unexpectedly, inhibition is more complete the more actively the leaf would otherwise have produced virus; not only is virus production decreased proportionally more in such conditions by thiouracil, but less total virus is formed than in leaves that are physiologically less capable of producing virus. Experiments with various viruses and hosts have further shown that thiouracil can affect the behaviour of healthy and infected plants in many ways that are not apparent when tobacco leaves recently inoculated with tobacco mosaic virus are floated in nutrient solutions.

MATERIALS AND METHODS

The viruses and host plants used were: tobacco mosaic virus (TMV), tomato aucuba mosaic virus, the Rothamsted culture of tobacco necrosis virus (RTNV), potato virus *Y*, and henbane mosaic virus in tobacco (*Nicotiana tabacum* L. var. White Burley); TMV and tomato bushy stunt virus in *N. glutinosa* L; RTNV in French bean (*Phaseolus vulgaris* L. var. Prince), and broad bean mottle virus in broad bean (*Vicia faba* L.).

Inocula were either purified virus preparations or sap freshly expressed from infected leaves. Inoculations were made by rubbing the whole upper surfaces of leaves as uniformly as possible with the forefinger wet with inoculum. When effects on virus production were studied, the inocula were either undiluted sap or purified TMV at 1 g./l.; when experiments involved counts of local lesions, the inocula were suitably diluted to give conveniently spaced lesions.

Thiouracil was mostly used as a solution of the potassium salt in water; comparable results were obtained with solutions of thiouracil made by warming suspensions in water. The effect of thiouracil on virus multiplication was mainly studied by floating whole leaves in solutions contained in shallow enamel dishes covered with glass sheets. Unless otherwise stated, the dishes were kept in the fluctuating conditions of a warmed glasshouse with a mean temperature of 20° and without artificial light. The solutions all contained 0·3 g. sulphonilamide/l., which does not affect virus multiplication but keeps the leaves and fluids free from bacterial growth; they usually also contained 10 g./l. sucrose and 0·2 g./l. $Ca(H_2PO_4)_2H_2O$, because leaves floated in this nutrient solution produce more TMV than do leaves floated in water (Kassanis, 1953). Leaves from 4 to 6 in. long were inoculated on the plants, washed with running water, and the plants were kept in the glasshouse for 24 hr., when the inoculated leaves were detached and floated. Leaves were not floated immediately after inoculation because this alone decreases the number of local infections obtained, and because thiouracil not only affects the rate of virus formation but can also prevent infection from occurring. Evidence for these two statements is given later.

Many experiments were also made with leaves left on the plants and kept under ordinary glasshouse conditions. In these, solutions of thiouracil were either sprayed over the leaves with a glass atomizer, or the thiouracil was added to the inoculum and rubbed over the leaves together with the viruses.

The relative virus contents of leaf extracts were usually measured serologically. Except with potato virus *Y* and henbane mosaic virus, which denature when heated and were therefore titrated from the fluids obtained by centrifuging sap at 8000 r.p.m., the leaf extracts were all heated to 60° before centrifuging. Heating not only coagulates the normal plant proteins and gives stable fluids for titration, but also aggregates the particles of anisometric viruses such as TMV and potato virus *X* and makes the precipitation titre a more reliable measure of virus content (Bawden & Pirie, 1945). The titrations were made by adding 1 ml. of the extracts at various dilutions to 1 ml.

of antiserum at a constant dilution of 1/100. The precipitation end-point was taken as the highest dilution at which a visible precipitate was produced after 3 hr. at 50° (40° with potato virus *Y* and henbane mosaic virus). The amount of TMV in extracts was sometimes measured by isolating and weighing the virus from a known volume of extract. In some experiments with TMV and RTNV the relative virus contents of extracts were estimated by infectivity tests, using the local-lesion method.

EXPERIMENTAL

Some effects of immersing inoculated leaves in water

All the viruses and hosts with which we have experimented give fewer local infections when inoculated leaves are immersed in water than when they are kept in air. The results of a typical experiment with TMV and *N. glutinosa* are given in Table 1. Two different treatments were used. Leaves were inoculated on the plants; for one treatment, they were cut length-wise along the mid-ribs, and one lot of half-leaves was left on the plant and the other submerged in water; for the other, the leaves were detached from the plants, and put vertically in tumblers with the mid-ribs at water-level, so that the lower halves were submerged while the upper halves were in air. Submersion in water decreases the numbers of lesions much more with the cut leaves than with the whole leaves. Whether this happens because the exposed halves of the whole leaves to some extent neutralize the action of submersion on the other halves, or because cut halves are more readily penetrated by water, is unknown.

Table 1. *The effect of submerging* Nicotiana glutinosa *leaves on numbers of local lesions produced by tobacco mosaic virus*

Treatment	Numbers of lesions per plant*			
A. Leaves cut into halves	Plant 1	Plant 2	Plant 3	Plant 4
(*a*) Halves left on the plants	345	671	270	246
(*b*) Halves submerged in water	96	222	88	70
Ratio *b* : *a*	0·28	0·33	0·33	0·28
B. Whole detached leaves	Plant 5	Plant 6	Plant 7	Plant 8
(*a*) Halves in air	216	524	493	359
(*b*) Halves submerged in water	91	233	302	169
Ratio *b* : *a*	0·42	0·44	0·61	0·47

* Eight leaves were inoculated on each plant, with TMV at 10 mg./l.

The extent to which submersion decreases the numbers of infections varies with the physiological state of the leaves. With *N. glutinosa* plants bearing eight to ten inoculable leaves, the oldest leaves are the most affected; when inoculated and submerged they may give only a tenth as many lesions as they would give in air, whereas submerging the youngest leaves may not decrease the numbers of lesions by as much as one half. With all the viruses and hosts we have tested, the effect of submersion decreases with increasing time after inoculation and is not appreciable after a day. With RTNV in French bean submersion 3–4 hr. after inoculation is usually ineffective, but with TMV in *N. glutinosa* infections are still inhibited by submersion 6–8 hr. after inoculation. No doubt the time at which the effect ceases to operate will vary, not only with individual viruses and host plants, but also with the temperature and many other factors. A simple explanation of the phenomenon would be that, for a time after inoculation, virus particles are loosely bound and can be dissolved out of the wounded cells. That this is unlikely to be the whole explanation, however, is suggested by the fact that the time after inoculation during which submersion decreases numbers of lesions is considerably longer than the time taken for rubbed leaves to regain their resistance to infection when sprayed with virus solutions (Kalmus & Kassanis, 1945). Also, submersing inoculated leaves in the virus solutions used as inocula decreases the numbers of lesions as much as does submersion in water, suggesting that virus particles cannot freely enter and leave the wounded cells.

From experiments comparing the numbers of local lesions produced by dry and wet inocula on wilted and sprayed leaves, Yarwood (1952) concluded that ‘water is injurious to the early phases of the infection process with certain viruses’. His treatments differ so widely from ours that there may be no common phenomenon. As leaf cells contain so much water, however, it seems unlikely that water would directly inhibit infection. Of the possible indirect actions, decreasing the supply of oxygen seems the most obvious, particularly as exposing recently inoculated leaves to atmospheres containing 50 % CO_2 also decreases numbers of local lesions (Kalmus & Kassanis, 1944). We have no positive evidence that some oxidation process is concerned, and bubbling air through water in which inoculated leaves were submerged did not affect the numbers of local lesions produced. Whatever the mechanism, it operates against some process that occurs only in the early phases of infection, for submersion does not decrease the rate at which virus is produced in infected leaves. On the contrary, leaves submerged a day after inoculation yield extracts containing more TMV or RTNV than do comparable leaves that are left on plants. Also, the local lesions that develop on submerged leaves, particularly those produced by RTNV in *N. glutinosa*, are often larger than those on leaves left on plants (Pl. 1, fig. 1).

Thiouracil and virus production

Table 2 shows how the yield of TMV obtained from inoculated leaves is increased when they are detached from the plant and floated. The differential treatments were started 24 hr. after the leaves were inoculated, and the leaves were macerated and the virus isolated from the extracts 9 days after starting the treatments. Leaves floated in water gave nearly twice as much virus as leaves left on the plants, and floating in a solution of sucrose and $Ca(H_2PO_4)_2$ produced nearly three times as much. Spraying leaves on the plants daily with thiouracil decreased the yield of virus, but less so than did floating in water containing thiouracil and much less so than floating in the nutrient solution containing thiouracil.

Derivatives of thiouracil seem not to interfere with the multiplication of

TMV, and floating tobacco leaves in nutrient solutions containing 100 mg./l. of either methyl-thiouracil or thiouracil-5-acetic acid gave as much virus as did floating leaves in nutrient solutions alone.

Table 2 also shows that uracil can completely counteract thiouracil's usual ability to inhibit virus production. This action seems specific to uracil; when adenine, cytosine or guanine were added to solutions of thiouracil, the amount of TMV produced was not significantly greater than that in leaves floated in solutions containing only thiouracil. Mercer, Lindhorst & Commoner (1953) also found this to be so, and they showed further that only uracil counteracts the ability of thiocytosine, thiothymine and two purine analogues, to inhibit the accumulation of TMV.

Table 2. *Concentration of tobacco mosaic virus in sap from inoculated tobacco leaves treated in different ways*

Treatment of leaves	Virus concentration (g./l. of sap)
Sprayed with water on plants	0·36
Sprayed with 100 mg. thiouracil/l.	0·14
Floated in water	0·55
Floated in 100 mg. thiouracil/l.	0·10
Floated in 100 mg. thiouracil/l. and 100 mg./l. uracil	0·50
Floated in nutrient solution	1·01
Floated in nutrient solution and 100 mg. thiouracil/l.	0·05
Floated in nutrient solution and 100 mg. thiouracil and uracil/l.	1·10

Inoculum was purified virus at 1 g./l. Nutrient solution contained 10 g./l. sucrose and 0·2 g./l. $Ca(H_2PO_4)_2H_2O$.

Commoner & Mercer (1952) found no qualitative differences between TMV formed in leaves floated in solutions with and without thiouracil. When we have purified TMV from treated and untreated leaves, we also have noticed no differences in general physical, chemical and serological behaviour, and, weight for weight, virus from treated leaves was as infective as that from untreated ones. When we assayed extracts from treated and untreated leaves both serologically and by infectivity tests, the two methods always gave comparable results. It seems, therefore, that thiouracil does not alter significantly the proportions in which infective and non-infective particles normally occur, but we have not attempted to fractionate the extracts by differential centrifugation and so have no critical experiments that would detect minor changes (Bawden & Pirie, 1945).

We have found that thiouracil inhibits TMV production most when conditions are such that without it most virus would be produced. Not only is the proportional decrease in virus greatest in such conditions, but less total virus is formed in the presence of thiouracil when conditions favour virus production than when they are less favourable. Of the conditions we have used, maximum virus is produced when inoculated leaves are floated in solutions of sucrose and $Ca(H_2PO_4)_2$ and exposed to daylight. Table 3 shows that in these conditions thiouracil inhibited the production of TMV almost completely, whereas it was almost ineffective with leaves floated in water and kept in darkness. We do not know whether this is because such leaves contain more uracil. In experiments we have made at different times with leaves floated in water, the amount of virus obtained in extracts has varied widely. The variations presumably reflect different physiological states of the leaves at the time they were floated, perhaps their sugar and phosphorus contents. Whatever the cause of the variations, the ability of thiouracil to inhibit TMV formation usually also varied and was small when the control leaves produced little virus.

Table 3. *The varying ability of thiouracil to inhibit the multiplication of tobacco mosaic virus in leaves treated differently*

	Relative virus content of sap*			
	Leaves in water		Leaves in nutrient solution	
A. Leaves in daylight	Water only	64*	Nutrient only	192
	Thiouracil 100 mg./l.	8	Thiouracil 100 mg./l.	1
B. Leaves in darkness	Water only	48	Nutrient only	128
	Thiouracil 100 mg./l.	32	Thiouracil 100 mg./l.	2

* Relative virus contents expressed as the reciprocal of the precipitation end-points when titrated against TMV antiserum. Leaves macerated 6 days after floating.

Table 4 shows that thiouracil can slow the multiplication rate of TMV at any time between infection and maximum virus content, although its inhibiting effect is less when virus has already multiplied considerably than it is when the treated leaves contain little virus. The leaves were detached at various times after inoculation, and floated in nutrient solutions, with and without thiouracil at 100 mg./l., for 7 days before they were macerated and their virus contents estimated. To find how much virus was produced before the differential treatments were started, some leaves were macerated at the times the others were floated. The leaves floated 12 days after inoculation were young leaves that had become infected by systemic spread of the virus, and thiouracil also decreased the rate at which TMV was produced in these.

When leaves are floated in solutions of thiouracil within a day or two after inoculation, they usually show no external symptoms, though sometimes they become rather more chlorotic than do comparable leaves floated in water or nutrient solutions. By contrast, when the interval between inoculation and floating is longer and the virus has already multiplied detectably before the leaves are exposed to thiouracil, they develop necrotic lesions, the size and distribution of which depend on the virus content of the leaves. Table 4 indicates this phenomenon, which is described in more detail later.

Exposing infected leaves to thiouracil does not inactivate virus already formed, nor does it have any permanent effect on the ability of the cells to maintain virus multiplication. The speed with which leaves floated in solutions of 100 mg. thiouracil/l. regained ability to produce TMV when they were washed in running water and then floated in water or nutrient solutions, varied considerably from experiment to experiment. Sometimes the washed leaves started to produce virus in the first day, whereas at others they took 2 or 3 days before the rate of virus multiplication was comparable with that in untreated leaves. We have not studied the reasons for this variation, but they may be correlated with the physiological condition of the leaves, which, as we have already shown, affects the extent to which thiouracil inhibits virus formation.

Table 4. *The effect of thiouracil applied at different times after tobacco leaves were infected with tobacco mosaic virus*

Days between inoculation and start of treatment	Treatment*	Relative virus content (Reciprocals of precipitation end-points)	Necrotic lesions
1	Thiouracil	1	—
	Nutrient solution	128	—
3	Sample before treatment	8	—
	Thiouracil	8	Small rings
	Nutrient solution	256	—
6	Sample before treatment	16	—
	Thiouracil	48	Rings
	Nutrient solution	512	—
9	Sample before treatment	128	—
	Thiouracil	256	Large rings and lines along the veins
	Nutrient solution	512	—
12	Sample before treatment	128	—
	Thiouracil	128	Patterns along basal veins
	Nutrient solution	512	—

* Detached leaves were floated in solutions of 10 g. sucrose/l. and 0·2 g. $Ca(H_2PO_4)_2H_2O$/l. with and without 100 mg. thiouracil/l., and were left for 7 days before being macerated. Some of the leaves detached at 3, 6, 9 and 12 days were macerated to find their virus contents when the treatments were started. The leaves floated after 12 days were uninoculated young leaves, becoming systemically invaded.

Thiouracil does not act specifically against TMV. On the contrary, it inhibits the multiplication in tobacco leaves of all the viruses we have tested. Results with three are shown in Table 5: tobacco leaves were detached and floated the day after they were inoculated, and they were macerated 1 week later, when the virus contents of the extracts were assayed serologically. The multiplication of potato virus *X* is also inhibited when inoculated leaves on the plant are sprayed daily with 100 mg./l. solutions of thiouracil. As with TMV, spraying has less effect than floating in solutions of thiouracil; in experiments when tobacco leaves floated in nutrient solutions, or left on the plant and sprayed with water, gave extracts with precipitation end-points of 1/128 to 1/256, extracts from leaves sprayed with thiouracil gave end-points of 1/8 or 1/16, whereas those from leaves floated in thiouracil failed to precipitate or did so only when undiluted. Experiments in which RTNV and tomato aucuba mosaic virus were inoculated to opposite halves of the same tobacco leaves, which were floated a day later, have sometimes suggested that thiouracil decreases the numbers of local lesions formed by tomato aucuba mosaic virus more than those formed by RTNV. However, as Table 6 shows, the multiplication of RTNV in tobacco leaves is also greatly inhibited. Tobacco leaves floated in nutrient solution a day after they were inoculated with RTNV gave extracts 6 days later that were 25 times as infective as extracts from comparable leaves floated in 100 mg./l. solution of thiouracil.

Table 5. *Thiouracil and the multiplication of four viruses in detached leaves*

		Reciprocals of serological titres	
Virus	Host	Leaves in thiouracil 100 mg./l.	Leaves in water
Potato virus *X*	Tobacco	0	160
Potato virus *Y*	Tobacco	0	8
Henbane mosaic	Tobacco	0	16
Broad bean mottle	Broad bean	32	32

Table 6. *Thiouracil and the multiplication of a tobacco necrosis virus in tobacco and French bean*

		No. of lesions* with sap diluted			
Host plant		1/625	1/125	1/25	1/5
Bean	Thiouracil	32	60	138	—
	Control	19	78	118	—
Tobacco	Thiouracil	—	20	112	206
	Control	102	195	555	—

* Total number of lesions on six French-bean leaves.

The ability of thiouracil to prevent virus formation does not extend to all hosts. Table 6 shows that RTNV multiplies equally in French-bean leaves floated in nutrient solutions with thiouracil as in solutions without it. Similarly, Table 5 shows that thiouracil has no effect on the multiplication of broad bean mottle virus in broad bean. This is so despite the fact that the inoculated leaves, which normally show no symptoms, develop large chocolate-coloured lesions after 4 days in the thiouracil solutions. Uninfected leaves in thiouracil solutions develop no such lesions.

Inhibition of infection and effects on host plants

Many substances, of widely different chemical constitutions, can affect the ability of plant viruses to cause infections. Characteristically, such inhibitors have no permanent effect on the viruses when incubated with them *in vitro*, and infectivity is fully affected immediately the inhibitors are added to inocula. How they act is unknown, but the extent to which some of the most powerful inhibitors decrease infectivity depends on the species of plant inoculated, whereas it is largely independent of the kind of virus. Thus, it seems likely that they affect some host-cell components with which the viruses need to interact during the initiation of infection.

Table 7 shows that thiouracil can be added to the list of substances that decrease the infectivity of virus preparations, and that it does so both in tobacco and French bean. To prevent the occurrence of infection, the concentration of thiouracil in inocula needs to be greater than that needed to decrease the rate of virus multiplication in floated tobacco leaves infected with TMV. As with trichothecin (Bawden & Freeman, 1952), spraying leaves with thiouracil before or after they are inoculated with viruses decreases the numbers of local lesions. Spraying inhibits infection more in tobacco than in French bean (Table 8), and in both species fewest lesions occur when the leaves are sprayed near to the time of inoculation. In tobacco, possibly because in this host thiouracil also has the additional ability to interfere with virus multiplication in infected leaves, spraying as long as 2 days after inoculation with tomato aucuba mosaic virus still decreases the number of lesions produced.

Table 7. *Thiouracil as an inhibitor of infectivity*

Concentration of thiouracil in inoculum (mg./l.)	Tobacco mosaic virus in *N. glutinosa*	Tobacco necrosis virus in French bean	Tomato aucuba mosaic virus in tobacco
2500	0*	0	2
625	10	11	9
156	137	199	48
39	210	219	106
0	189	487	132

* Total number of lesions from nine leaves.

Table 8. *Inhibition of infection by spraying with thiouracil*

Interval between spraying and inoculation	Tomato aucuba mosaic virus in tobacco	Tobacco necrosis virus French bean
48 hr. before	3*	12†
24 hr. before	2	12
1 hr. before	0·3	8
24 hr. after	3	19
48 hr. after	10	26
Unsprayed control	27	29

* Average number of lesions on 16 leaves. † On 36 leaves.

The experiments in which leaves left on plants were exposed to thiouracil, either contained in the inoculum or sprayed as separate solutions, showed effects of thiouracil that are not apparent when mature leaves are floated in solutions. With French bean, for example, the first-formed leaves were used for local-lesion counts and for experiments on the multiplication of RTNV. When floated in 100 mg./l. solutions of thiouracil, such leaves show few or no effects. Nor are they much affected when rubbed or sprayed with the concentrated solutions of thiouracil used to inhibit infection. The young trifoliate leaves of these plants, however, even though they are not directly treated with thiouracil, become almost completely yellow, an effect that persists for some days, when they regain their normal green. Spraying uninfected broad-bean plants with thiouracil produces little response, but it affects plants inoculated with broad-bean mottle virus. The inoculated leaves develop chocolate-coloured local lesions (Pl. 1, fig. 2), similar to those on infected leaves floated in solutions of thiouracil, and instead of the mottle that normally develops on young leaves when systemically invaded, these and the stem apex become black and die.

Matthews (1953*a*) found that spraying tobacco plants with thiouracil turns the young leaves yellow, makes their edges curl, and checks apical growth. We have confirmed this observation. When sprayed daily with 100 mg. thiouracil/l., the young leaves develop chlorotic areas on the fourth day and start to curl. Sprayed with 1 g./l. solutions, the young leaves are completely bleached after 4–6 days. While spraying is continued, the plants make little or no growth, but when it stops they gradually recover and after a week or more show few effects. When tobacco plants, recently inoculated with TMV on their lower leaves, are sprayed daily with thiouracil solutions, the young leaves become chlorotic as in uninfected plants, but the inoculated leaves behave differently (Pl. 1. fig. 3). They develop necrotic spots or rings, the number of which depends on the virus content of the inoculum and the size on the time that has elapsed between inoculation and spraying (Pl. 1, fig. 4). Spraying a day after inoculation causes small necroses to appear within 3 or 4 days. As the delay between inoculation and spraying is progressively increased, so the spots and rings become increasingly larger. This fits with what is known of the manner in which viruses spread from the initial infection sites, and it suggests that the necrosis is a reaction to thiouracil of cells in which virus is actively multiplying. The customary method whereby the local lesions of TMV in tobacco are made visible is to detach the leaves, decolorize them with ethanol and stain with iodine (Holmes, 1931). Spraying with thiouracil solutions is a simpler technique and has the added advantage that the leaves are not detached and killed, but we have not tested its usefulness for local-lesion counts. The interaction between thiouracil and infected cells that leads to necrosis is not restricted to inoculated leaves. When young leaves are floated in or sprayed with thiouracil at the time they are becoming systemically invaded and would normally become yellow around the veins, the yellowing in parts of the leaves is replaced by necrosis. Young leaves with the mosaic symptoms typical of a full systemic infection may develop a few isolated necrotic areas, but their main reaction is to become yellow so that the mosaic disappears. It seems that the necrotic rings and patterns are mainly a response of recently infected cells in which the virus is multiplying rapidly. A simple explanation would be that infected cells contain no free uracil, but spraying plants with mixtures of uracil and thiouracil does not prevent the necrosis. Nor does spraying with such mixtures prevent thiouracil from producing its usual chlorosis on young leaves of tobacco and French-bean plants.

Matthews (1953*a*) found that spraying guanazolo on *N. glutinosa* leaves inoculated with lucerne mosaic virus considerably delayed the time taken for plants to become systemically infected, and he used the delay to measure the extent to which the substance inhibited virus multiplication. Spraying thiouracil over tobacco leaves recently inoculated with TMV also delays the onset of systemic infection, the length of the delay depending on the interval between inoculation and spraying and on the strength of the solutions sprayed. Spraying with concentrated solutions immediately after inoculation may prevent systemic infection, but spraying immediately with 100 mg. thiouracil/l., or spraying with concentrated solutions a day or more after inoculation, does not prevent the young leaves from ultimately becoming infected. The concentrated solutions applied immediately after inoculation presumably prevent infection from being initiated (Table 7), whereas after the virus has started to multiply, although thiouracil slows the rate of multiplication, it does not stop it completely or inactivate the virus already formed. Delay in the development of systemic symptoms is not a convenient method of studying the inhibiting action of thiouracil, for the young leaves on sprayed plants become so chlorotic, and the new growth is so abnormal, that the initial symptoms are difficult to detect. When spraying is stopped, apical growth is resumed and infected plants gradually develop the usual symptoms. Sap from leaves of such plants contains as much virus as sap from comparable leaves of unsprayed plants.

DISCUSSION

In discussing the ability of thiouracil to inhibit the rate of TMV formation, Commoner & Mercer (1952) say 'the simplest, but by no means the most likely explanation of this effect, is that thiouracil prevents the entry of uracil into TMV nucleic acid and thereby forestalls the formation of the virus particle. Alternatively, it is possible that thiouracil blocks the participation of uracil in some process more remotely related to the TMV particle.' Our results do not decide definitely between these two; nor, indeed, are they the only possibilities. For example, Matthews (1953*b*) found that guanazolo, which also inhibits virus formation in *Nicotiana* sp., although less thoroughly than does thiouracil (Mercer *et al.* 1953), is incorporated in the nucleic acid of TMV formed in treated tobacco leaves, and he suggests that virus formation may be inhibited because such particles are 'sterile'. Similarly, Jeener & Rosseels (1953) state, in a preliminary note, that thiouracil can be incorporated in the nucleic acid of TMV, and they also suggest that this modification may hinder multiplication.

The inhibitory action of thiouracil on virus formation cannot yet be interpreted with any certainty, and discussion is worth while only in as far as it indicates further lines of work. An obvious one is to find whether virus particles containing thiouracil or guanazolo do differ biologically from normal particles. The fact that anomalous substances can be incorporated in virus particles has great intrinsic interest, but virus particles are unlikely to be the only components of cells to which such things happen, and its relevance to the problem of virus multiplication will remain uncertain until the chemically modified particles are shown to multiply more slowly or to interfere with the multiplication of normal particles. Also uncertain is the relevance of what happens when thiouracil is having relatively little effect on virus multiplication, the conditions under which it has been found to be incorporated in TMV, to what happens when it is inhibiting so strongly that very little virus is formed. The small amount of TMV extractable from tobacco leaves that are floated a day after inoculation in nutrient solution containing thiouracil, is certainly not all non-infective, and our tests did not suggest that its ability to initiate new infections in *N. glutinosa* differed greatly from that of normal virus. However, although infectious towards *N. glutinosa*, the particles might not be able to multiply in tobacco cells containing thiouracil. Alternatively, the infective particles could be normal ones, produced in parts of cells incompletely invaded by thiouracil, and if these were more readily extractable than those modified by thiouracil, then the modified and possibly non-infective particles would be selectively retained in the leaf residues.

Another line of work that might profitably be pursued is the comparative study of tobacco and French-bean leaves, to try and find reasons for the ability of thiouracil to inhibit virus formation in one but not the other. Is RTNV containing thiouracil able to multiply in French bean but not in tobacco? Or is thiouracil not incorporated in viruses when they are being formed in French bean? Or is the prime effect of thiouracil on some host component?

The results of spraying plants with thiouracil show that it does disturb their metabolism, producing particularly obvious effects in young tissues where continued protein synthesis is needed, and it is reasonable to assume that effects on virus formation may be correlated with this disturbance. Failure of some host-cell mechanism is reasonably suspected, too, from the fact that what specificity there is in the ability of thiouracil to inhibit virus formation seems to be determined by the identity of the host plant and not by the virus. Thus, although in tobacco thiouracil seems to act indiscriminately and inhibit the formation of any virus including RTNV, it does not affect the multiplication of RTNV in French bean. Effects on the external appearance and on the growth of host plants, however, need not be directly related to effects on virus production. Indeed, there are indications that the two are separate. Uracil, which neutralizes thiouracil's ability to inhibit virus multiplication in tobacco, does not neutralize its effects on young leaves and on apical growth. Also, when French-bean plants are sprayed with thiouracil, the young leaves react in a manner superficially similar to tobacco leaves, showing that host-plant metabolism can be affected without affecting the ability to support virus multiplication. One explanation of these differences could be that some system concerned with protein synthesis in tobacco, but not in bean, is adversely affected by thiouracil. A nucleic acid is the obvious suspect, but there is little call to speculate on the nature of a substance whose existence is unestablished. Another and simpler explanation would be that French-bean leaves contain more uracil than do tobacco leaves and so are better able to neutralize the power of thiouracil to inhibit virus formation. We have not attempted to assess the relative uracil contents of the different plants, but preliminary paper chromatograms suggest that this would not be difficult. Our qualitative tests were made with sap from tobacco leaves only, and they show that the synthesis of TMV in this host does affect the contents of uracil and of other purines and pyrimidines. Uracil was easily detected in sap from healthy tobacco leaves after it was freed from protein and nucleic acid. It was also detected in sap from leaves that had been inoculated with TMV and treated with thiouracil so that virus multiplication was largely inhibited, whereas it was not detected in sap from untreated leaves in which the virus had multiplied extensively. Thus the multiplying virus normally seems to sequestrate the free uracil, but the inability of virus to multiply in the presence of thiouracil seems not to be attributable to a loss of uracil. How extra uracil counteracts the inhibiting power of thiouracil is unexplained, but it may prevent the incorporation of thiouracil in either the virus or some host nucleic acid, or both.

We are indebted to Dr P. P. Hopf, of Ward, Blenkinsop and Company, Ltd., for gifts of uracil, thiouracil and its derivatives; also to Dr A. Kleczkowski who made the chromatograms.

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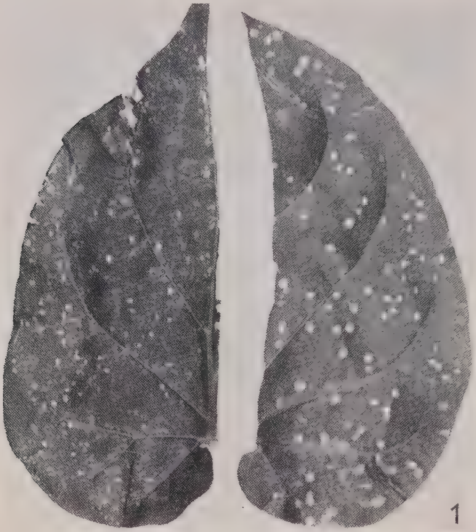

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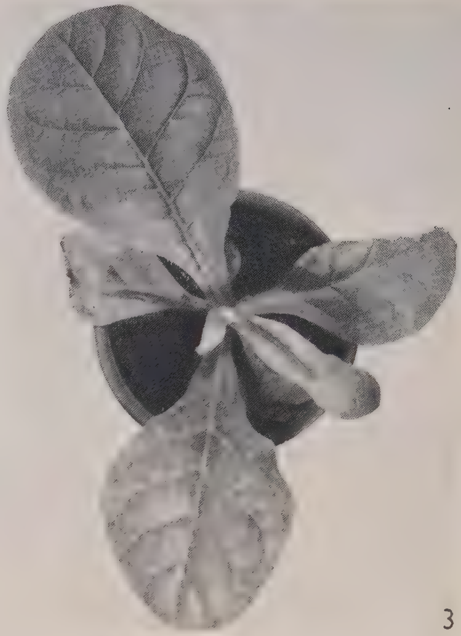

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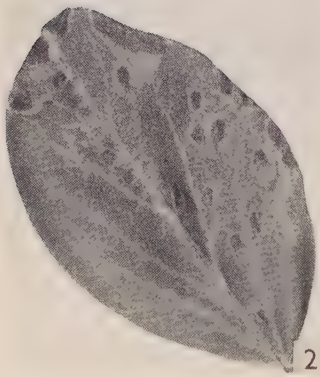

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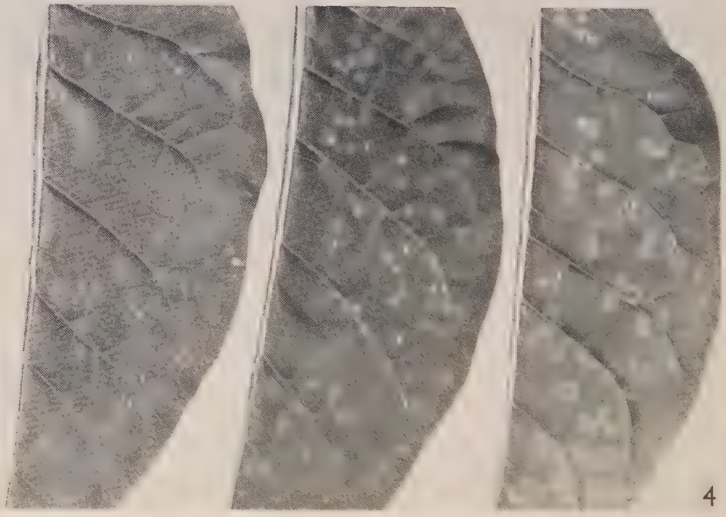

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F. C. Bawden & B. Kassanis—Thiouracil and plant viruses. Plate 1

EXPLANATION OF PLATE

Fig. 1. Half leaves of *Nicotiana glutinosa* inoculated with Rothamsted tobacco necrosis virus, showing (left) the small lesions normally produced, and (right) large lesions when leaf is floated in water.

Fig. 2. Leaflet of *Vicia faba* inoculated with broad bean mottle virus showing the necrotic lesions developed by spraying with thiouracil.

Fig. 3. Tobacco plant inoculated with tobacco mosaic virus and sprayed with thiouracil. The inoculated leaves have many necrotic lesions and the young leaves are chlorotic and curled.

Fig. 4. Half leaves from tobacco plants sprayed with thiouracil at different times after inoculation with tobacco mosaic virus. Note the increase in size of necrotic lesions with increasing time between inoculation and spraying. Left-hand half-leaf sprayed 1 day after inoculation; centre 2 days and right-hand 3 days.

(*Received* 13 *August* 1953)

COWAN, S. T., SHAW, C. & WILLIAMS, R. E. O. (1954). *J. gen. Microbiol.* **10**, 174–176.

Type Strain for *Staphylococcus aureus* Rosenbach

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SUMMARY: A strain isolated from a human source is described and is proposed as the type for the species *Staphylococcus aureus* Rosenbach in place of the one chosen by Shaw, Stitt & Cowan (1951) which has some characters of animal strains.

The nomenclatural type concept in bacteriology dates from the selection of the Marburg strain of *Bacillus subtilis* as the type strain of that species (St John-Brooks & Breed, 1937). Rule 9 of the bacteriological code (Buchanan, St John-Brooks & Breed, 1948) states that 'For each valid name of each taxonomic group there shall be designated a type; that is for each species or subspecies a type culture, specimen or description, for each genus a type species (genotype).' It is proposed (Editorial Board, 1953) that this rule shall be amended to indicate more precisely how the type strain shall be chosen when the original author of a name did not preserve the culture on which he based his description. A substitute type culture (neotype culture) should be one accepted by international agreement; 'It should agree with the diagnosis given by the original describer, and should be recommended by a Committee of workers familiar with the species and approved by the Judicial Commission' (Editorial Board, 1953, p. 43, draft proposal 14).

Rosenbach (1884) adopted Ogston's (1882) generic name *Staphylococcus*, and named two species, *Staphylococcus aureus* and *Staphylococcus albus*, both based on organisms isolated from infective processes in man. He did not choose a type culture, and we do not know of the existence of any of Rosenbach's original cultures. Consequently, when Shaw, Stitt & Cowan (1951) reviewed the classification and nomenclature of staphylococci, they selected as type a strain, FDA 209-P, maintained in the American Type Culture Collection (ATCC 6538P) and in the National Collection of Type Cultures (NCTC 7447). Gibson (1953) has asked for an official Opinion of the Judicial Commission on the validity of the generic name *Staphylococcus*, and has proposed that the culture FDA 209-P should be recognized as the type culture of *Staphylococcus aureus* Rosenbach.

Before designating it as a type strain Shaw *et al.* (1951) examined the cultural characters of FDA 209-P, and chose it because: (1) it was well-pigmented and on subculture retained its ability to produce pigment; (2) its biochemical characters were fairly typical of the species; (3) the strain was in use as a reference strain for penicillin assay and was held in at least two of the major bacterial culture collections. Recently an attempt was made to classify the strain by bacteriophage and serological methods; it was found to be bacteriophage type 42D, and serologically untypable. Bacteriophage type 42D is associated with infections of cattle, and it seems probable that FDA 209-P is not of human origin.

We think that the neotype culture of *Staphylococcus aureus* should be a strain isolated from a pathological lesion in man and that it should have characters associated with other human strains. Consequently we withdraw our earlier choice of FDA 209-P as the neotype culture of *Staphylococcus aureus* and propose in its place strain $S33R_4$, a description of which follows.

DESCRIPTION OF PROPOSED NEOTYPE CULTURE $S\,33\,R_4$

History of the strain. Isolated on 13 November 1935, from a specimen of human pleural fluid which contained acid-fast bacilli. Maintained by subculture in nutrient agar stabs and passaged through four rabbits before preservation in the dry state in March 1939. Deposited in the National Collection of Type Cultures in May 1953 (NCTC 8532).

Metabolism. The strain is an aerobe and facultative anaerobe. It grows equally well at 30 and at 37°. It is not able to utilize citrate as the sole carbon source, or ammonium salts as the sole nitrogen source.

Morphology. The organisms are Gram-positive, spherical or near spherical in shape, $0{\cdot}7$–$1{\cdot}0\,\mu$ in diameter, and arranged in pairs or small grape-like clusters. They have not been found to be motile, and they do not spore.

Colony form. After 24 hr. at 37° on Lemco agar colonies are 0·5–1·0 mm. in diameter. They have a low convex elevation, a smooth shining surface and an entire edge. When left at room temperature in diffused daylight colonies increase in size and become coloured with a rich cream or gold pigment. The colonies are opaque, butyrous in consistency, and the growth is easily emulsifiable. On agar containing 5% (v/v) horse blood, colonies are surrounded by clear zones of haemolysis.

Broth culture. After 24 hr. at 37° the medium shows a dense general turbidity and a powdery deposit, moderate in amount. With continued incubation the bulk of the deposit increases and a ring pellicle forms on the surface.

Gelatin stab. Growth along the needle track shows the first signs of liquefying the medium after 24 hr. at 22°. At 48 hr. there is saccate liquefaction extending to the limit of the inoculation.

Bromcresol purple milk. Acid after 1 day, clot after 5 days at 37°. Slight digestion of clot begins about the 7th day.

Biochemical reactions. The methyl red and Voges-Proskauer tests are positive after 5 days at 30°. At 37° ammonia is produced from peptone, but neither H_2S nor indole is produced from media containing adequate amounts of sulphydryl compounds and tryptophan; nitrates are reduced to nitrites; catalase is produced on Lemco agar; starch is not hydrolysed; urease is produced. The strain is phosphatase-positive (Barber, Brooksbank & Kuper, 1951). On egg-yolk agar at 37° (Gillespie & Alder, 1952) a slight opacity is produced around the colonies. Inspissated serum is not digested within 7 days at 37°.

Fermentation reactions. At 37° acid is produced from glucose, xylose, lactose, sucrose, maltose, glycerol and mannitol, but not from dulcitol or salicin.

Special characters. The coagulase produced by this strain clots human and rabbit plasma. α-Toxin is produced in an atmosphere of 20% (v/v) CO_2. The strain is sensitive to penicillin, streptomycin, aureomycin and chloramphenicol, and is resistant to sulphathiazole. Serologically it is type III (Cowan, 1939), of which it is the type strain.

The strain is lysed strongly by the following bacteriophages of the standard set used in the routine typing method described in the appendix to the report of Williams, Rippon & Dowsett (1953): 6, 7, 47, 54, 75. It is, in addition, lysed strongly by two other phages not now ordinarily used in routine typing, namely 75A and 75B. When tested with undiluted filtrates the strain is lysed strongly by the following phages of the standard set: 6, 7, 42E, 47, 53, 54, 70, 73, 75, 77; and also by the following additional phages: 42B, 42F, 47B, 47C, 52B, 57, 58, 75A, 75B, 76, 78.

The strain is a typical member of the group III defined by Williams *et al.* (1953).

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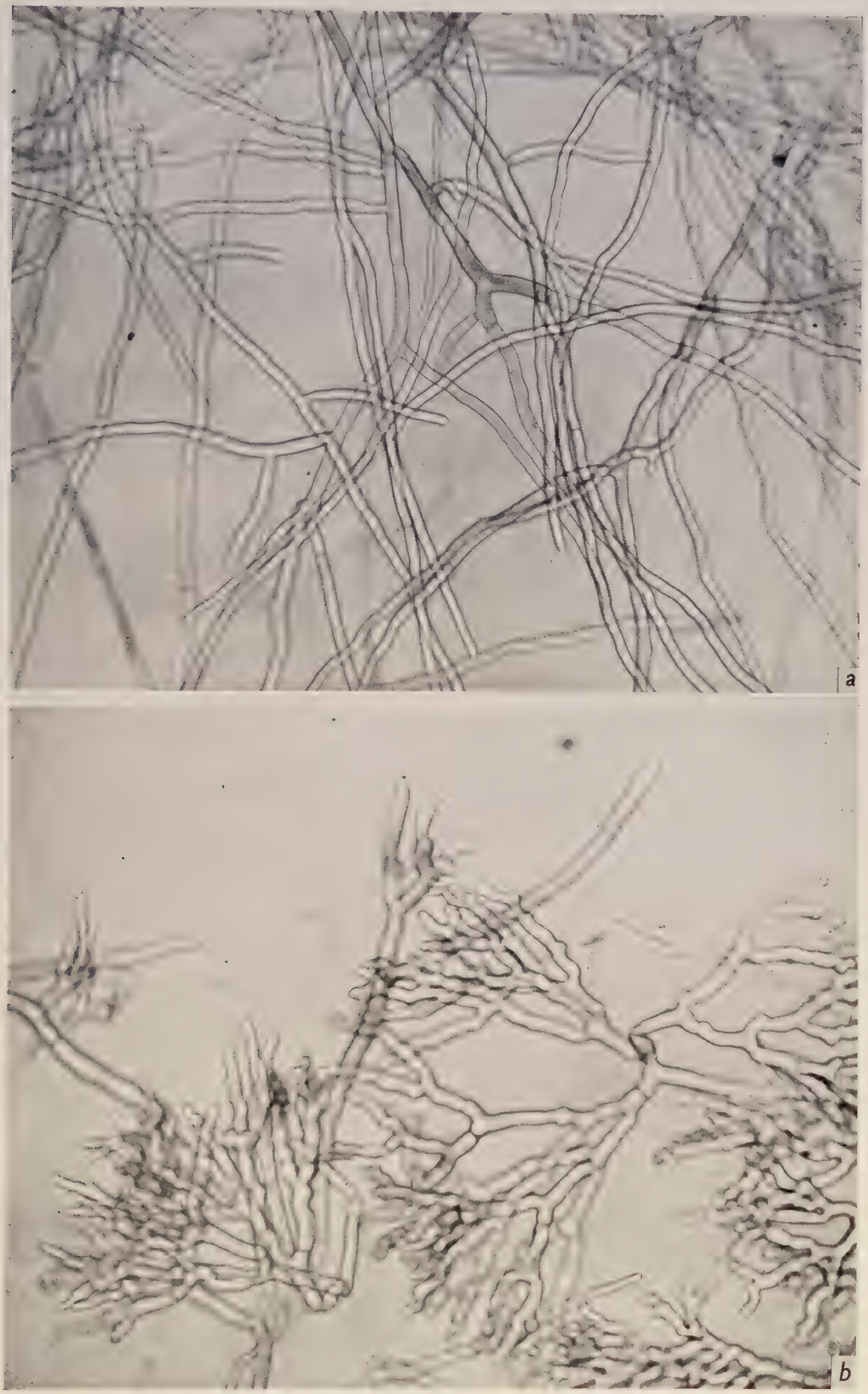
Journal of General Microbiology, Vol. 10, *No.* 1



H. A. Painter—Factors affecting growth of sewage fungi. Plate 1

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Factors affecting the Growth of some Fungi associated with Sewage Purification

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SUMMARY: Various aspects of the nutrition of four fungi which commonly inhabit percolating filters treating sewage have been studied. *Sepedonium* n.sp., a predominating fungus, required organic sources of nitrogen while *Fusarium aqueductum*, *Geotrichum* sp., and *Trichosporon cutaneum* were able to utilize ammonium salts; *F. aqueductum* was also able to utilize nitrate. *Geotrichum* sp. and *F. aqueductum* needed no external supply of vitamins, *T. cutaneum* required thiamine and *Sepedonium* sp. required thiamine and biotin for optimum growth. The pH range for optimum growth was narrow (pH 7 to 8·5) for *Sepedonium* sp. while the ranges for the other fungi were much wider (pH 3 or 4 to 9).

Zn, Ca, Mn and probably Fe and Cu were required by *Sepedonium* sp., which was the only one of the four fungi inhibited by concentrations of Zn above the optimum in media partially deficient in other elements. Mn, Fe or Cu when added with Zn counteracted the inhibitory action, while Ca increased it.

The purification of sewage by filtration through material such as gravel is due largely, if not solely, to the activity of organisms in the film present on the material of the filter. The film consists of algae, fungi and bacteria, together with amorphous matter derived from sewage. Excessive growth of the film, especially at the surface, may result in a decrease in the efficiency of the filter by impeding the percolation of sewage through the filter (causing 'ponding') and also by interfering with aeration. Fungi play an important part in causing ponding since the fibrous and intractable nature of the film is due largely to the presence of fungal mycelium (Tomlinson, 1942). Tomlinson & Hall (1950) found that from 5 to 30% of the total solids in the film in eight small-scale filters was of fungal origin. Thus, if the growth of fungi were limited, the tendency of the filter to pond would be lessened while the efficiency of the process would probably not be impaired. For treating trade wastes unmixed with sewage, on the other hand, it may become desirable to promote the growth of fungi in order to purify the waste.

Little is known about the nutritional requirements of fungi encountered in sewage purification, and it was thought that such knowledge might be of use in the problem of fungus control. This paper describes experiments concerning the growth requirements of four sewage fungi. The effects on *Sepedonium* sp. of comparatively high concentrations of some trace elements are also described.

METHODS

Organisms. The fungi used were chosen because of their widespread occurrence in filters at many sewage works. Three of the organisms were identified by the Centraal Bureau at Baarn as *Fusarium aqueductum* Lagerh., *Trichosporon* *cutaneum* (de Beurm., Gougerot et Vaucher) and *Geotrichum* sp. (considered to be synonymous with *Oospora*). The fourth, which was isolated by Tomlinson (1942), was provisionally identified as a new species of *Sepedonium* by Dr Mason of the Commonwealth Mycological Institute.

Stock cultures were grown on glucose Yeastrel agar slopes and stored at room temperature. Except in the case of the new species of *Sepedonium* the inocula consisted of spores scraped with a platinum loop from the surface of cultures 1–2 weeks old. *Sepedonium* sp. was first subcultivated on 1 % Bacto-agar plates for 5–15 days, the inocula consisting of small pieces of agar containing a few hyphal fragments. Experiments have established that negligible carry-over of nutrients resulted from these methods of inoculation.

Composition of media. Medium A contained (g./l.): glucose (Difco) 5·0; vitamin-free Casamino acids (Difco), 5·0; KH_2PO_4, 0·5; $MgSO_4.7H_2O$, 0·5; KCl, 0·25. Water obtained by one distillation from a metal still was used.

Medium B was prepared by adding 2 ml. of solution E to every litre of medium A. Solution E, based on a modification of Hoaglands 'A to Z' mixture (Robbins & Kavanagh, 1938), was prepared by mixing separate solutions of salts of eleven trace elements immediately before use. The final concentrations of the elements in medium B were (in p.p.m.): Ca (as $CaCl_2$) 1·0; Zn (as $ZnSO_4$) 0·4; Fe (as $FeSO_4$) 0·4; Cu (as $CuSO_4$) 0·2; Mn (as $MnSO_4$) 0·2; B (as H_3BO_3) 0·2; Mo (as $(NH_4)_6Mo_2O_7$) 0·1; Ni (as $NiCl_2$) 0·1; Co (as $Co(NO_3)_2$) 0·1; Br (as KBr) 0·1; I (as KI) 0·1.

The media were adjusted, in most experiments, to pH 7 with NaOH; in some experiments adjustment to other pH values was made with NaOH or H_2SO_4.

A solid medium was prepared by mixing one part of quadruple-strength medium A, containing any substance being tested, and three parts of silica sol, prepared by the method of Taylor (1950), together with sufficient NaOH to neutralize the sterilized sol. The three components were sterilized separately by autoclaving at 15 lb./sq.in. for 15 min. and were mixed immediately before pouring into Petri dishes.

The concentrations of vitamins used were (μg./l.): biotin, 20; thiamine hydrochloride, pyridoxine hydrochloride, nicotinamide, folic acid, riboflavin, calcium pantothenate, *p*-aminobenzoic acid, 80; inositol, choline chloride, 1000. The liquid media were sterilized by autoclaving at 15 lb./sq.in. for 15 min. after the addition of substances under test.

Removal of trace elements from media. It is well known that because of their powers of chelation it is difficult to free amino acids from trace elements. In preliminary experiments some measure of success was achieved in removing trace elements from medium A by repeated extraction with 8-hydroxyquinoline (oxine) as described by Chesters & Rolinson (1951). As Donald, Passey & Swaby (1952) reported that a medium containing peptone could be at least partially purified by autoclaving with chromatographic Al_2O_3 (5 g./l.) at pH 7·5 (their method 5), this method was applied to medium A. The other method used was no. 13, as listed by Donald *et al.* (1952), in which the medium was repeatedly extracted with oxine and then with dithizone.

Conditions of cultivation. All glassware was cleaned in chromic + sulphuric acid mixture, thoroughly rinsed with tap water, then with distilled water and in appropriate cases, with double-distilled water. 100 ml. conical flasks (Hysil) containing 25 ml. medium were used. The flasks were fitted with cotton-wool plugs or with metal caps (Oxoid). All cultures were incubated in the dark at 20° a temperature which Tomlinson (1942) had shown to be about optimum for the growth of *Sepedonium* sp. After incubation, for 10 days unless otherwise stated, the fungal mats were collected on sintered-glass crucibles (porosity no. 1), washed with distilled water, and dried for several hours at 100°. Filtration of cultures of *Sepedonium* sp. and *F. aqueductum* was rapid, but that of *Geotrichum* sp. and *T. cutaneum* was much slower. At least three flasks were used in each group and the means of the weights are recorded together with, in some cases, the standard deviations.

Sewage. Fresh settled sewage was filtered and, when necessary, its pH was adjusted to 7; its initial pH was usually 7·5–8. It was then used without further treatment or was first concentrated tenfold *in vacuo* with a still temperature of about 60°.

Ashed sewage. Fresh settled sewage was filtered and the organic matter was destroyed either by evaporating to dryness and heating to redness after addition of HNO_3 or by repeated evaporation with HNO_3 on a steam bath. The residue in each case was dissolved in a little concentrated HCl, with heating. The solution was then diluted to a convenient volume with distilled water and the pH value adjusted to 7. A slight flocculent precipitate sometimes resulted and was dispersed by stirring immediately before the liquid was added to the culture medium. The two methods of destroying organic matter gave identical biological results.

RESULTS

Growth rates

The rates of growth of the four fungi were determined in medium B supplemented with vitamins. There was a lag period of 1–2 days except with *Sepedonium* sp., the lag period for which was 4 days. Maximum growth resulted after about 10 days with this fungus while the other fungi reached maximum growth after about 7 days.

Effect of hydrogen ion concentration

The relations between the initial pH value of the medium and growth of the four fungi are given in Fig. 1 (*a*–*d*). Medium B supplemented with vitamins was used and the incubation period was 11 days, except for *Sepedonium* sp. which was incubated for 13 days. *F. aqueductum* and *T. cutaneum* were both capable of good growth over the wide pH range 4–9, and both tolerated pH values beyond this range. *Geotrichum* sp. also tolerated a wide range (pH 3–10) and grew well over the range pH 3–9; best growth resulted at pH 3 though no growth occurred at pH 2. In contrast, *Sepedonium* sp. grew well only within narrow limits, pH 7–8·5, although it tolerated the pH range 4–10. The pH values of those acid media in which growth had occurred had risen by varying degrees at the end of the incubation period while the pH values of alkaline media had fallen to about 8.

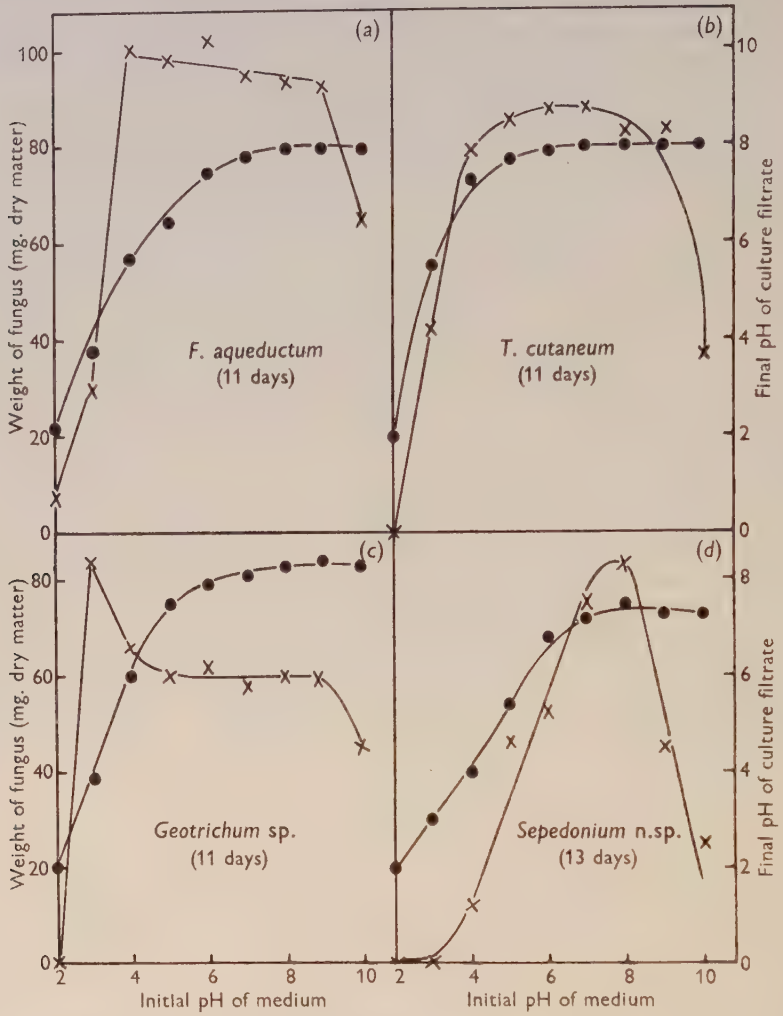

Fig. 1. Relation between pH value of medium and growth of fungus. Each point is the mean of four determinations. —×—×—, weight of fungus; —●—●—, final pH value of medium.

The assimilation of various sources of nitrogen

The suitability of different sources of nitrogen for the four fungi was determined using medium B, containing thiamine and biotin but omitting Casamino acids, as the basal medium. The various substances containing nitrogen were added in amounts such that each medium contained 0·035 % N (equivalent to 0·5 % vitamin-free Casamino acids). The yields of fungal mycelium are given in Table 1 which shows that *Sepedonium* sp. grew best on complex organic sources of nitrogen and grew very poorly when the nitrogen was provided by nitrate or ammonium sulphate, confirming the findings of Tomlinson (1942). *Geotrichum* sp. and *T. cutaneum* were able to utilize ammonium sulphate in addition to organic nitrogen, while *F. aqueductum* was able to utilize nitrate as well as the other forms of nitrogen. Best growth of all four fungi resulted when the source of nitrogen was Casamino acids. Other experiments in which *Sepedonium* sp. was cultivated on silica-gel media containing amino acids singly and in various combinations showed that the fungus did not preferentially utilize any of the eleven amino-acids tested; best growth resulted on the medium containing all eleven.

Table 1. *The availability of various sources of nitrogen to four fungi*

Nitrogen compounds added in amounts sufficient to give a concentration of 0·035 % N. Weight of *Sepedonium* n.sp. determined after 13 days; that of other fungi after 11 days. Each figure is the mean of four determinations.

	Weight of fungus (mg. dry wt./25 ml. medium)			
Source of nitrogen	*F. aqueductum*	*T. cutaneum*	*Geotrichum* sp.	*Sepedonium* n.sp.
None	4	2	2	5
KNO_3	48	3	1	7
$(NH_4)_2SO_4$	35	55	38	12
Glycine	59	54	19	12
Asparagine	64	50	22	41
7 amino acids*	—	59	33	42
17 amino acids†	96	68	32	40
'Casamino acids'	99	83	53	73
Bacto-peptone	66	52	24	68

* These were: glycine, cystine, glutamic acid, valine, leucine, proline and threonine.

† These were the above seven, together with ten others known to be constituents of casein.

Vitamin requirements

An indication of the vitamin requirements of the four fungi was first obtained by assessing visually their growth on medium B, solidified with silica gel, to which were added singly and in combinations ten members of the B-group vitamins. The results indicated that while *F. aqueductum* and *Geotrichum* sp. were independent of an external supply of vitamins, *T. cutaneum* required thiamine and *Sepedonium* sp. required some other factor or factors in addition to thiamine.

The requirements of the first three organisms were confirmed by results of experiments, given in Table 2, using liquid cultures with medium B as the basal medium. The growth of *Geotrichum* sp. and *F. aqueductum*, whether determined after 6 or 9 days, was not influenced appreciably by the vitamin content of the medium. *T. cutaneum* grew well only when thiamine was present while addition of other vitamins with thiamine did not further increase the yield. It can also be seen from Table 2 that *Sepedonium* sp. requires both thiamine and biotin. Thiamine alone resulted in poor growth while the presence of the other eight B-vitamins in addition to thiamine and biotin produced no further increase in growth.

Table 2. *The influence of various B-group vitamins on the growth of four fungi*

Weight of *Sepedonium* sp. determined after 12 days; that of other fungi after 6 days. Each weight given is the mean of three determinations. Standard deviations are given in brackets.

	Weight of fungus (mg. dry wt./25 ml. medium)			
Additions to vitamin-free medium	*F. aqueductum*	*Geotrichum* sp.	*T. cutaneum*	*Sepedonium* n.sp.
None	87 (4·2)	51 (10)	6 (0·7)	10 (1·0)
Thiamine	90 (4·0)	53 (5·5)	93 (4·0)	18 (1·4)
Biotin	83 (7·2)	60 (5·2)	6 (0·7)	13 (6·1)
Pyridoxin	90 (3·5)	62 (3·5)	6 (0·7)	12 (2·0)
Thiamine + biotin	87 (4·1)	61 (4·0)	87 (7·2)	74 (3·6)
Thiamine + pyridoxin	86 (3·2)	64 (4·2)	93 (3·1)	18 (2·1)
Biotin + pyridoxin	84 (3·7)	50 (2·5)	8 (1·6)	17 (2·6)
9 members of B group omitting thiamine	90 (4·0)	59 (5·0)	6 (2·0)	21 (3·0)
9 members of B group + thiamine	88 (3·7)	65 (6·7)	86 (4·1)	71 (2·6)

The concentration of thiamine which gave maximum growth of *T. cutaneum* and *Sepedonium* sp. under the conditions employed was *c.* 1 μg./25 ml.; half maximum growth was produced by *c.* 0·1 μg./25 ml. No inhibition resulted when the concentration was as high as 10 μg./25 ml.

Two other types of Sepedonium, isolated from other filters, were found to have the same requirement as the original type. A fourth type, isolated from the River Dee, grew poorly on all media used.

Table 3. *The requirement of* Sepedonium *sp. and* T. cutaneum *for the moieties of thiamine*

Each weight given is the mean of five determinations. Standard deviations are given in brackets.

	Weight of fungus (mg. dry wt./25 ml. medium)	
Additions to biotin-supplemented basal medium	*Sepedonium* n.sp. (after 10 days)	*T. cutaneum* (after 7 days)
None	14 (2·8)	12 (1·7)
Thiazole*	12 (1·1)	13 (0·5)
Pyrimidine†	16 (5·2)	10 (0·5)
Thiazole + pyrimidine	97 (4·0)	86 (3·0)
Thiamine	96 (3·5)	85 (4·2)
Sulphite-treated thiamine	31 (1·2)	49 (4·5)
Sulphite-treated thiamine + thiamine	95 (5·3)	84 (1·3)

* 4-Methyl-5-(β-hydroxyethyl)-thiazole.

† 2-Methyl-4-amino-5-aminomethyl pyrimidine dihydrochloride.

The results in Table 3 show that *Sepedonium* sp. and *T. cutaneum* require both the thiazole and pyrimidine moieties of the thiamine molecule for growth; neither moiety alone sufficed. A mixture of the two moieties was as good as thiamine itself when synthetic substances were used, but when thiamine hydrolysed by treatment with sulphite (Fitzgerald & Hughes, 1949) was used, yields much less than the maxima were obtained. Thus the sulphite treatment (autoclaving at 10 lb./sq.in. for 10 min. at pH 5·4) gave products which were not readily available to the fungi as sources of thiamine.

Minor element requirements of Sepedonium *sp.*

The requirements of *Sepedonium* sp. for trace elements were ascertained by determining the effects on growth of deficiencies of six elements in turn. The elements were added in concentrations probably somewhat above optimum to medium A which was supplemented with thiamine and biotin and which had been treated to remove trace elements. It was found that the trace elements were not removed to the same extent each time by either method used; Stockdale (1953) experienced similar, but greater, variations when she applied the $CaCO_3$ method of Steinberg (1919) to a peptone medium. On some occasions in the present work the removal of trace elements as a whole was at least as good as that achieved by Donald *et al.* (1952) when they applied the Al_2O_3 method to 5% sucrose peptone water with *Aspergillus niger* as test organism.

Table 4. *Growth of* Sepedonium *n.sp. in medium treated by two methods to remove trace metals*

Complete medium contained Fe, Zn, Cu, Ca, Mn, and Mo. The elements were added at the following concentrations (p.p.m.): Fe, Zn, 2; Cu, Mn, 1; Ca, 25; Mo, 0·5. Each weight is the mean of five determinations. Standard deviations are given in brackets.

Treatment of medium	Dry weight of mycelium in complete medium (mg./25 ml. medium)	Metal deficiency						
		All 6	Fe	Zn	Cu	Ca	Mn	Mo
		Weight of fungus in deficient media (% of growth in complete medium)						
Extraction with oxine and dithizone	88 (9·8)	14 (8·5)	93 (9·3)	57 (10·3)	73 (6·0)	27 (12·5)	28 (7·1)	97 (15·0)
Al_2O_3 method	91 (2)	1 (0·7)	70 (5·5)	52 (9·2)	96 (11·5)	14 (8·7)	31 (4·0)	95 (4·5)

From Table 4, which contains the results of experiments using each of the two methods described, it may be concluded that *Sepedonium* sp. requires Zn, Ca and Mn and also probably Fe and Cu; at no time was a requirement for Mo established. Except in the case of Mn, the deficiencies of individual elements were not as complete as those obtained by the above authors. A probable reason for this is the comparative difficulty in removing the last traces of these metals from media (such as those used in the present work) containing amino acids, which were absent from the medium used in the studies of individual element requirements of *A. niger* (Donald *et al.* 1952). Each of the other fungi grew better when trace elements were added to the untreated medium; the requirements for individual elements were not determined.

Relation between concentration of minor elements and growth

Some indication of the optimum concentration of trace elements was obtained by determining the yields of *Sepedonium* sp. in untreated medium A to which was added from 0 to 500 ml. solution E/l. The growth response was roughly logarithmic up to 50 ml. solution E/l. (Fig. 2). Growth was completely inhibited at 500 ml./l., which produced a fairly heavy precipitate in the medium. By extrapolation it was estimated that the particular batch of basal medium used produced the same effect as about 0·1 ml. solution E/l.

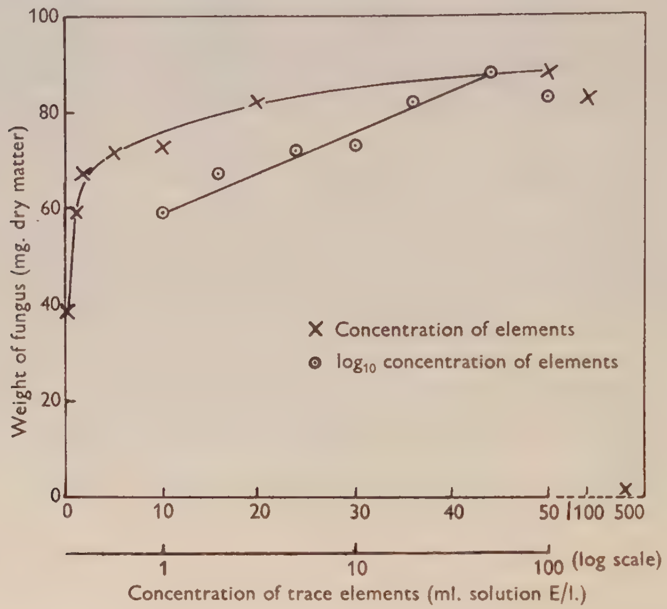

Fig. 2. Influence of the concentration of trace elements on the growth of *Sepedonium* n.sp. in untreated medium. 1 ml. solution E/l. gave the following concentrations (p.p.m.): Ca, 0·5; Fe, Zn, 0·2; Cu, Mn, 0·1; Co, Mo, Ni, Br, I, 0·05.

Since preliminary experiments showed that untreated medium A was apparently deficient only in Zn and Ca it was of interest to ascertain the effects on the growth of the fungus of various combinations of different concentrations of these two elements. Fig. 3(*a*) shows the growth response of *Sepedonium* sp. in vitamin-supplemented, untreated medium A to increasing concentrations of Zn under different conditions, namely: (i) with no further addition; (ii) with the addition of 10 p.p.m. Ca; (iii) with the addition of 10 p.p.m. Ca and nine other elements at concentrations equivalent to 20 ml. solution E/l. Fig. 3(*b*) shows the responses when the roles of Zn and Ca were reversed, 4 p.p.m. Zn being added in appropriate cases.

The results show that Zn was required at a concentration of 0·5–1·0 p.p.m., and Ca was required at the comparatively high concentration of 5–12·5 p.p.m. In the absence of other added elements, concentrations of Zn greater than the optimum inhibited growth; this inhibition was enhanced in the presence of 10 p.p.m. Ca. The addition of up to 25 p.p.m. Ca alone did not produce inhibition.

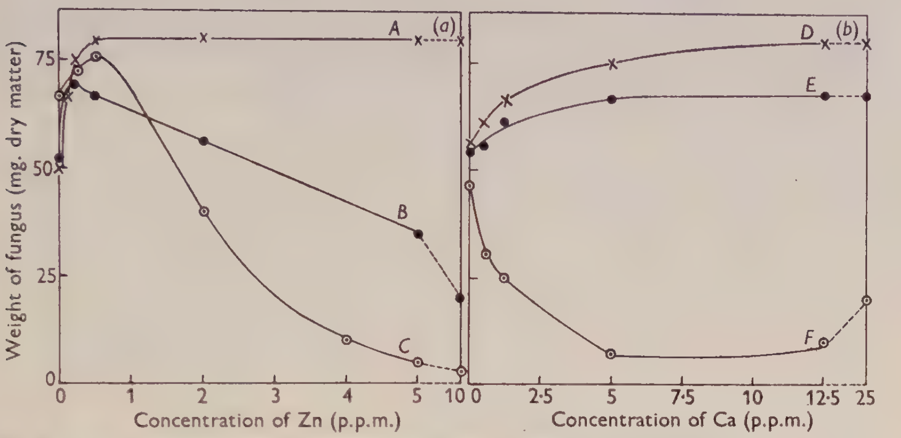

Fig. 3. Effect on the growth of *Sepedonium* n.sp. of high concentrations of (*a*) zinc, and (*b*) calcium added to untreated medium. Elements added: A, Ca and nine other elements at concentrations equivalent to the addition of 20 ml. of solution E/l.; B, none; C, 10 p.p.m. Ca; D, Zn and nine other elements at concentrations equivalent to 20 ml. of solution E/l.; E, none; F, 4 p.p.m. Zn.

Effects of high concentrations of minor elements

The effect of high concentrations of other elements was ascertained by adding them singly and in pairs to untreated medium A at the following concentrations (p.p.m.): Fe, Cu, Mn, Zn, 10; Mo, 5; Ca, 25. The results (Table 5) show that complex relations exist between the metals. Zn and Fe may be regarded as primary agents in causing inhibition under these conditions since only these two metals, when added singly, resulted in yields lower

Table 5. *Effect on the growth of* Sepedonium *n.sp. of the addition to untreated medium of six metals, singly and in pairs, in relatively high concentrations*

Each weight given is the mean of three determinations. Standard deviations are given in brackets. The metals were added at the following concentrations (p.p.m.): Mo, 5; Zn, Fe, Cu, Mn, 10; Ca, 25.

	Element added					
	Zn	Ca	Fe	Cu	Mn	Mo
Element added	Weight of fungus (mg. dry wt./25 ml. medium)					
Zn	3 (3·1)	1 (0·7)	57 (17·0)	79 (12·5)	85 (3·6)	39 (16·8)
Ca	—	72 (4·1)	46 (3·5)	74 (1·7)	74 (1·3)	75 (3·1)
Fe	—	—	26 (3·8)	17 (3·1)	47 (3·5)	29 (1·7)
Cu	—	—	—	45 (0·7)	72 (5·0)	58 (7·5)
Mn	—	—	—	—	67 (4·0)	71 (1·0)
Mo	—	—	—	—	—	48 (5·3)

Weight of fungus with no addition of metals = 45 mg. (4·0). Weight of fungus with addition of all six metals = 83 mg. (2·1).

than the control (the yield in untreated medium containing no added trace elements but which contained trace elements as impurities). Mn and Cu completely prevented and Mo partially prevented the inhibition produced by Zn, while in contrast the addition of Ca had no effect. The combination of Zn and Fe resulted in more growth than was produced by either element alone. The inhibition caused by Fe, which was not so large as that caused by Zn, was increased by the presence of Cu, was unaltered by Mo, and was decreased by Mn and Ca.

In other experiments in which the concentrations of the elements were smaller (namely (p.p.m.): Fe, Cu, Mn, Zn, 4; Mo, 2; Ca, 10) it was found that the only appreciable inhibition was produced by the combination of Zn and Ca, either element alone having no inhibitory effect. It was also found that the growth-depressing effect of 10 p.p.m. Zn and 25 p.p.m. Ca could be completely overcome by the initial addition of as little as 2 ml. of solution E/l. Normal growth was also restored when the trace element solution was added to a 10-day-old inhibited culture. There was no similar inhibition of growth of the other fungi in the presence of 10 p.p.m. Zn and 25 p.p.m. Ca as was observed with *Sepedonium* sp.; *F. aqueductum* continued to grow well even at 100 p.p.m. Zn.

Cultures of *Sepedonium* sp. containing inhibitory concentrations of Zn and Ca not only grew feebly but also presented an appearance different from normal growth. Whereas the fungus normally grew in a continuous mat over the whole surface of the liquid, the Zn-inhibited cultures grew in small submerged islets which were whiter and denser than normal growth. Microscopical examination revealed that Zn had induced a greater degree of hyphal branching and vacuolization (Pl. 1, fig. *a*). Cultures of the fungus inhibited by 10 p.p.m. Fe or 10 p.p.m. Fe and 10 p.p.m. Cu presented much the same appearance as normal cultures.

Growth of Sepedonium *sp. in the presence of sewage*

Little information is available concerning the presence of vitamins in settled sewage, although it is to be expected that, because of its origin, it would contain members of the B group unless they had been destroyed or otherwise removed during the passage of sewage through the sewers and through the primary sedimentation tanks at a disposal works. Rudolfs & Heinemann (1940) showed that sewage contains substances which stimulate the growth of yeast, and Kraus (1942) established the presence of a factor in sewage which behaves like riboflavin. More recently Hoover, Jasewicz & Porges (1951) demonstrated the presence of cobalamin in sewage sludge, and the presence of nicotinamide and pantothenic acid has also been reported ('H.J.S.' 1952).

A rough assessment of the thiamine content of sewage was made by determining the yields of *Sepedonium* sp. obtained in thiamine-free media to which were added varying amounts of sewage or concentrated sewage. Medium B, supplemented with biotin, was used as the control medium; the results are given in Table 6. Since it had been shown that 1 μg. thiamine/25 ml. produced maximum growth and 100 ml. sewage/25 ml. usually produced maximum growth, it may be inferred that the sewages tested contained thiamine in a concentration of the order of 1 μg./100 ml. In other experiments in which biotin was omitted from the control medium the fungus grew well when sewage was added, indicating the presence of biotin in sewage.

Table 6. *Growth of* Sepedonium *n.sp. in medium containing sewage as the sole source of thiamine*

	Volume of sewage present in 25 ml. medium (ml.)			
	0	10	50*	100*
	Weight of fungus (mg. dry wt./25 ml. medium)			
Average of 6 means, each of 3 determinations	6	30	63	86
Range of means	2–8	19–43	35–80	51–105

* Added in the form of tenfold concentrated sewage.

To obtain some idea of the trace element content of sewage the yields of *Sepedonium* sp. were determined in medium A supplemented with thiamine and biotin and containing increasing amounts of sewage. Table 7 shows that the smallest quantity of trace elements necessary for maximum growth was contained in 2–10 ml. sewage. When the experiment was repeated with re-dissolved ashed sewage (Table 7), maximum growth occurred in the same range of volume but the fungal yield was about 10 % less than that obtained with whole sewage. This small difference, which was statistically significant at the 10 ml. level, occurred with all the samples of sewage tested, whether solely domestic or containing trade wastes.

Table 7. *Influence of the amount and form of sewage on the growth of* Sepedonium *n.sp. in untreated medium containing the necessary vitamins but no added trace elements*

Each weight given is the mean of six determinations. Standard deviations are given in brackets.

	Volume of sewage present in 25 ml. medium (ml.)					
	0	1	2	10	20*	50*
Form of sewage	Weight of fungus (mg. dry wt./25 ml. medium)					
Untreated	48 (7·7)	62 (5·4)	80 (7·1)	105 (3·5)	105 (5·2)	105 (4·0)
Ashed	48 (7·7)	60 (5·5)	76 (4·0)	90 (5·5)	90 (4·1)	—

* Added in the form of tenfold concentrated sewage.

This point was investigated in more detail by comparing the rates of growth of the fungus in a medium to which trace elements had been supplied by adding sewage (10 ml./flask), ashed sewage (≡10 ml. sewage/flask), or solution E (20 ml./l.). It can be seen from Fig. 4, which illustrates a typical set of results, that the growth curves obtained with solution E and with ashed sewage were identical within the limits of experimental error. The medium containing whole sewage produced a higher growth rate and a higher maximum yield (10–15 % higher) than either of the other two media. This small stimulatory effect could not be accounted for although a number of possible factors have been studied.

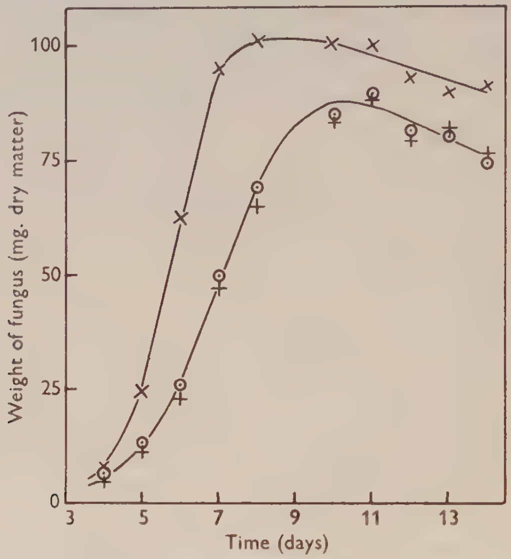

Fig. 4. The influence on the growth of *Sepedonium* n.sp. of sewage, ashed sewage, and trace elements added to untreated medium containing added vitamins. —×—×, 10 ml. sewage/25 ml. medium; —⊙—⊙, 10 ml. sewage, ashed/25 ml. medium; —+—+, trace elements, 20 ml. solution E/l.

DISCUSSION

Whereas Zn, Mn, Fe and Cu shown here to be required (or probably required) by *Sepedonium* sp., have frequently been reported to be necessary for the growth of fungi, Ca has rarely been reported to be essential. Using very refined methods, Steinberg (1948) established that the growth of various fungi was inhibited to varying degrees in Ca-deficient media. In those cases where Ca was required (four or five out of seven), 2–6 p.p.m. gave optimum growth; this compares with 5–12·5 p.p.m. required by *Sepedonium* sp. More recently, Basu (1951) has shown that Ca stimulates the vegetative growth, as well as the fruiting, of some species of *Chaetomium*, the optimum concentration being in the region of 10 p.p.m. Taylor (1951) found that Ca stimulated the growth of certain soil bacteria, but was of opinion that it acted as an antagonist to some toxic substance in the peptone of his medium.

A point of interest arising from the results is that *Sepedonium* sp. is sensitive to comparatively low concentrations, 4–10 p.p.m., of Zn when grown in media partially deficient in other trace metals. True comparison with the results of other workers is difficult because of the differences in the trace element content of the basal media employed in the different investigations. Studies of this type have almost completely been confined to fungi which can utilize inorganic sources of nitrogen and consequently the media used have been more easily freed from trace elements than the media containing organic nitrogen used in the present work.

Zn has frequently been reported to be toxic to fungi, but usually the concentrations involved have been much higher than 4 p.p.m. For example, Gollmick (1936) found that 1000 p.p.m. Zn completely prevented the growth of *Aspergillus niger* in the presence of 1 p.p.m. Fe and 1 p.p.m. Cu while 500 p.p.m. Zn did not. Roberg (1928), however, claimed that as little as 1–5 p.p.m. Zn inhibited the growth of *A. niger* in the absence of Fe, which when added with Zn removed the inhibition. This action was attributed by Roberg to antagonism but another explanation, put forward by Foster (1939), is that the organism required both Zn and Fe. The latter view is supported by the fact that growth in the control medium containing no added metals was either the same as, or less than, that obtained when 1–5 p.p.m. Zn was added. In the case of *Sepedonium* sp. there seems to be an antagonistic action not only between Zn and Fe but also between Zn and Mn and between Zn and Cu. This is thought to be so because the mycelial yield produced by the addition of 10 p.p.m. Zn was considerably less than that obtained with the partially deficient basal medium alone and because the addition of Fe, Mn, or Cu with Zn increased the yield to values greater than the control. It is to be pointed out that the growth of the fungus on the basal medium appeared to be normal and the yield, which was in the region of 50 % of that obtained when the deficiencies were made good, was increased by the addition of Zn in concentrations up to about 2 p.p.m.

The effect of Ca also merits attention. While the addition of 4 p.p.m. Zn or 10 p.p.m. Ca to the basal medium did not result in any decrease in growth, the simultaneous addition of the elements at these concentrations lowered the yield almost to nothing. Mann (1932) observed a similar depressant effect by much higher concentrations of Ca on *A. niger*, for which Ca is not essential. She found that 500 p.p.m. Ca lowered the yield produced by 0·65 p.p.m. Zn by 35 % and 8000 p.p.m. lowered it by 46 %.

Increased branching of the hyphae and vacuolization in the hyphae caused by concentrations of Zn above optimal, as described here for *Sepedonium* sp., were observed by Iwanoff (1904) in *A. niger* and four other fungi. The concentrations used by this worker were considerably higher, namely 130–65,000 p.p.m.

Finally, it may be mentioned that the differences in growth rates and nitrogen requirements between *F. aqueductum* and *Sepedonium* sp. offer an explanation of the common observation that the former organism appears before the latter on the surface of percolating filters newly put into operation. This is supplementary to the explanation given by Tomlinson (1942) who showed that the undifferentiated thallus of *Sepedonium* sp., unlike that of *F. aqueductum*, is not adapted to adhere to a surface.

This paper is published by permission of the Department of Scientific and Industrial Research. Mr T. G. Tomlinson isolated the organisms, took the photographs and provided useful discussion and criticism. Miss D. H. M. Jordan and Mr R. A. Varley assisted with the experiments.

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EXPLANATION OF PLATE

Plate 1

(*a*) Hyphae of *Sepedonium* n.sp. grown in vitamin-supplemented Medium A containing 4 p.p.m. Zn, 10 p.p.m. Ca and nine other elements at concentrations represented by 2 ml. solution E/l. ×200.

(*b*) Hyphae of *Sepedonium* n.sp. grown in vitamin-supplemented medium A containing 4 p.p.m. Zn + 10 p.p.m. Ca ×200.

(*Received* 17 *August* 1953)

Jawetz, E., Gunnison, J. B. & Coleman, V. R. (1954). *J. gen. Microbiol.* **10**, 191–198.

Observations on the Mode of Action of Antibiotic Synergism and Antagonism

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SUMMARY: Inactive analogues and degradation products of active antibiotics failed to participate in either synergistic or antagonistic antimicrobial action in combination with active antibiotics. Streptobiosamine hydrochloride contaminated with 2–3 % (w/w) streptomycin was synergistic with other antibiotics because of the active streptomycin present. The ratio of the smallest amount of antibiotic participating in synergism to that having independent antimicrobial activity varied with the drug and the micro-organism. Synergism with another antibiotic usually required from 1/20 to 1/3 of the minimal inhibitory concentration of an antibiotic when acting alone. Streptomycin, however, in 1/1000 of the minimal inhibitory concentration was synergistic with penicillin in its action on a strain of *Streptococcus faecalis.*

The combined effect of two antibiotics upon a bacterial population may be greater or less than that of either alone. One manifestation of such combined action is an alteration of the early bactericidal rate. A marked increase in the rate of bactericidal action of a pair of antibiotics above that achieved by a single drug may be called 'synergism'; a marked decrease, 'antagonism'. The experimental basis of combined action and its application have been summarized recently (Jawetz & Gunnison, 1953). It has been postulated that antibiotic synergism and antagonism are the results of specific drug action on metabolic pathways which are essential for synthesis and growth. Antagonism has been observed only when the interfering antibiotic was used in a concentration having at least a bacteriostatic effect when acting alone (Gunnison, Coleman & Jawetz, 1950). Hence, it seemed unlikely that chemical analogues and degradation products of antibiotics lacking in antimicrobial effect would interfere with the active antibiotics. In synergism, on the other hand, only one of a pair of antibiotics need exhibit independent antimicrobial action at the concentration employed in the combination (Jawetz, Gunnison & Coleman, 1950; Chabbert, 1953). It was therefore possible that analogues and degradation products might enter into synergistic combinations even though themselves devoid of antimicrobial action. This paper records the results obtained with combinations of inactive analogues or degradation products and active antibiotics and also observations on quantitative aspects of synergism *in vitro.*

MATERIALS AND METHODS

Antibiotics and other compounds. The substances tested were: (*a*) chlortetracycline hydrochloride (aureomycin, Lederle) in freshly prepared solution, and in a form partially inactivated by heating 200 μg. aureomycin/ml. in 0·85 %

(w/v) sodium chloride solution at pH 8 at 72° for 13 hr. in a water-bath until less than 1/100 of the original activity remained; (*b*) bacitracin (Pfizer); (*c*) chloramphenicol (Parke, Davis) and a biologically inactive isomer from its D-base; (*d*) potassium penicillin G and the degradation products penicillamine hydrochloride and *iso*propyllidine penicillamine hydrochloride; (*e*) streptomycin sulphate and the degradation products streptamine dihydrochloride, streptidine sulphate, and streptobiosamine dihydrochloride; (*f*) oxytetracycline hydrochloride (terramycin, Pfizer); (*g*) erythromycin (Abbott). Stock solutions of these substances were prepared by dissolving weighed amounts in 0·85 % (w/v) sodium chloride solution. These were sterilized by Seitz filtration and stored at −20°. Final dilutions were freshly prepared in broth. All active antibiotics except bacitracin were pure crystalline materials and their activity is therefore expressed in μg./ml. The bacitracin preparation was impure and its activity is expressed in units. The penicillin preparation contained 1600 units/mg.

Media. Proteose peptone no. 3 (Difco) broth of the following composition was used: Proteose peptone no. 3, 20 g.; glucose, 0·5 g.; sodium chloride, 5 g.; disodium phosphate ($Na_2HPO_4 . 12H_2O$), 5 g.; water, 1000 ml.; pH 7·2. Proteose no. 3 agar (Difco) was used for plate cultures.

Organisms used. Klebsiella pneumoniae (strain AD), *Streptococcus faecalis* (strain 16) and *Staphylococcus aureus* (strains Heatley, De, Co, and Er) were used as test bacteria.

Procedure. Broth containing the diluted antibiotics, analogues or degradation products, alone or in combination, was inoculated with an 18 hr. broth culture of the test bacteria to give a concentration of 10^7 to 10^8 organisms/ml. in a total volume of 15 ml. Samples of 0·5 ml. were removed at frequent intervals during incubation at 37°. The number of viable bacteria was estimated by plate counts. Details of the method have been described (Gunnison, Jawetz & Coleman, 1950). Only differences in the number of bacteria greater than tenfold (1 log unit) were regarded as significant. The smallest amount of drug which resulted in a bacterial count significantly lower than that in the drug-free control in at least two successive determinations at any time during 30 hr. of exposure to the drug was designated as the 'minimal effective dose' (M.E.D.).

RESULTS

Biologically active aureomycin was antagonistic to penicillin when acting on *K. pneumoniae* (Gunnison, Coleman & Jawetz, 1950). The addition of bacteriostatic amounts of aureomycin (0·1–5·0 μg./ml.) to rapidly bactericidal amounts of penicillin resulted in a marked decrease in the bactericidal rate. Smaller amounts of aureomycin (0·01–0·05 μg./ml.), devoid of antimicrobial action, showed no such antagonism. Aureomycin inactivated by heating, in any concentration, likewise failed to interfere with the action of penicillin (Table 1).

Chloramphenicol interfered with the rapid lethal action of penicillin on many bacteria (Jawetz, Gunnison, Speck & Coleman, 1951). The biologically inactive isomer from the D-base showed no antagonism (Table 1). Chloramphenicol was synergistic with streptomycin in its action against *Staph. aureus*

Heatley, but the isomer from the D-base could not be substituted for the active chloramphenicol.

Penicillin and streptomycin were markedly synergistic against *K. pneumoniae* and *Strep. faecalis*. In these synergistic systems, penicillamine hydrochloride or *iso*propyllidine hydrochloride, both without antimicrobial effect, could not be substituted in any concentration for active penicillin.

Streptamine dihydrochloride and streptidine sulphate had no demonstrable bacteriostatic action and could not take the place of streptomycin in synergistic combinations with either penicillin or bacitracin acting on any of the test organisms (Table 1). Streptomycin was synergistic with terramycin against *Staph. aureus* De, but neither streptidine nor streptamine in any concentration could be substituted for the active streptomycin. Streptobiosamine hydrochloride, on the other hand, showed clear-cut synergism when combined with penicillin acting on *Strep. faecalis* or with bacitracin acting on *Staph. aureus* Heatley (Table 1). In concentrations up to 250 μg./ml., streptobiosamine alone was as completely devoid of antibacterial action against *Strep. faecalis* as were streptidine and streptamine. However, 25 μg. streptobiosamine/ml. in combination with 6 μg. penicillin/ml. gave as striking synergism against *Strep. faecalis* as did streptomycin itself. In several other systems, streptobiosamine showed an additive effect of lesser magnitude.

Although streptobiosamine alone in concentrations up to 100 μg./ml. had no demonstrable effect upon any of the test bacteria, in a dose of 250 μg./ml. it produced a slight transitory inhibition of the growth of *Staph. aureus* De, less than that shown by one M.E.D. of streptomycin. It was suspected, therefore, that it might contain traces of active streptomycin. Upon inquiry, Dr O. Wintersteiner, in whose laboratories the streptobiosamine had been prepared, stated that it was probably contaminated with 2–3 % (w/w) of active streptomycin. If this contamination were responsible for the marked synergism observed, then small amounts of streptomycin, far less than the M.E.D., must be capable of participating in synergistic combinations. Accordingly, quantitative tests were made of several synergistic pairs to ascertain how small a proportion of an independently effective amount of antibiotic might enter into synergism.

A variety of systems was tested in which synergism conforming to the following definition occurred: 'The addition of one drug to another results in a marked increase in bactericidal rate within the first 8–24 hr. of exposure *in vitro*, and the bactericidal rate of the combination is more rapid than twice the concentration of each single drug participating in the mixture' (Jawetz & Gunnison, 1953). In such systems, synergism regularly occurred when each member of the drug pair was present in a minimum effective dose (M.E.D.) which alone resulted in mere inhibition of growth (Table 2). Moreover, synergism also occurred when fractions of the M.E.D. of one antibiotic were combined with an M.E.D. of the other, i.e. an ineffective amount of one drug plus a barely active amount of the other might be synergistic (Table 2). Decreasing amounts of each drug were therefore combined with one M.E.D. of the other to determine how small a fraction might participate in synergism.

When penicillin and streptomycin acted on *K. pneumoniae*, there was no synergism when the concentration of either drug was below 1/3 M.E.D. (Table 2). Although a concentration of 5 μg. streptomycin/ml. participated in synergism, 1 μg./ml. failed to so do. With streptobiosamine, a concentration of 50 μg./ml., containing 1·0–1·5 μg. streptomycin/ml. as impurity, gave a slight additive effect with penicillin. Using *Staph. aureus* De as test organism, 1/5 M.E.D. of streptomycin (4 μg./ml.) or 1/4 M.E.D. of oxytetracycline were the smallest amounts resulting in synergism with 1 M.E.D. of the other drug (Table 2). When streptobiosamine was substituted for streptomycin, a concentration of 250 μg./ml., containing 5·0–7·5 μg./ml. of streptomycin, gave an additive effect, but 1/10 of this amount did not enter into combined action. These results, therefore, were consistent with the assumption that the activity shown by the streptobiosamine preparation was due to the streptomycin present.

Table 1. *Participation of analogues and degradation products of antibiotics in synergism and antagonism*

Test organism	Antibiotics or preparations thereof (μg./ml.)	Period after inoculation (hr.)				Combined effect
		0	2	7	24	
		Number of viable bacteria/ml.				
K. pneumoniae AD	None	2×10^7	10^8	5×10^8	9×10^8	—
	Penicillin 12·0		5×10^6	10^2	$<10^1$	—
	Chloramphenicol 1·0		7×10^6	8×10^6	10^7	—
	Pen. 12·0 + chlor. 1·0		10^7	2×10^5	7×10^3	Antagonism
	Inactive chlor. isomer 1·0		10^8	3×10^8	10^9	—
	Pen. 12·0 + chlor. isomer 1·0		2×10^6	3×10^2	$<10^1$	No antagonism
	Aureomycin 1·0		10^7	2×10^4	1×10^8	—
	Pen. 12·0 + aureo. 1·0		6×10^6	4×10^4	4×10^3	Antagonism
	Inactive aureo., heated 10·0		8×10^7	2×10^8	9×10^8	—
	Pen. 12·0 + aureo., heated 10·0		3×10^6	3×10^2	$<10^1$	No antagonism
Staph. aureus Heatley	None	9×10^6	6×10^7	2×10^8	$>10^9$	—
	Bacitracin 4·0*		8×10^7	5×10^6	4×10^4	—
	Streptidine 250·0		4×10^8	5×10^8	$>10^9$	—
	Baci. 4·0* + streptidine 250·0		3×10^7	5×10^6	9×10^3	No synergism
	Streptobiosamine 250·0		6×10^7	3×10^8	$>10^9$	—
	Baci. 4·0* + streptobios. 250·0		10^7	2×10^5	3×10^1	Synergism
Strep. faecalis 16	None	3×10^7	9×10^7	5×10^8	$>10^9$	—
	Penicillin 6·0		9×10^6	4×10^5	3×10^4	—
	Streptomycin 25·0		10^8	4×10^8	3×10^8	—
	Pen. 6·0 + strepto. 25·0		5×10^5	7×10^3	10^2	Synergism
	Streptobiosamine 250·0		10^8	6×10^8	$>10^9$	—
	Pen. 6·0 + streptobios. 250·0		2×10^6	3×10^4	7×10^2	Synergism

* In units/ml.

Similar dose relationships were observed in the following systems: bacitracin + streptomycin, acting on *Staph. aureus* Co (Table 2); bacitracin + erythromycin (Fig. 1) or bacitracin + chloramphenicol, acting on *Staph. aureus* Er; penicillin + bacitracin, penicillin + streptomycin, or bacitracin + streptomycin, acting on *Staph. aureus* Heatley; and bacitracin + streptomycin or penicillin + bacitracin acting on *Strep. faecalis*. In all these systems, fractions smaller than 1/20 to 1/3 of the M.E.D. of one drug failed to show synergism with one M.E.D. or more of the second drug.

Table 2. *Relationship between antibiotic concentrations giving independent inhibition of growth and concentrations able to participate in synergistic combinations*

Test organism	Antibiotics (μg./ml.)	Period after inoculation (hr.)				Minimum effective dose of single drugs	Combined effect
		0	2	7	24		
		Number of viable bacteria/ml.					
Staph. aureus Co	None	4×10^{7}	8×10^{7}	2×10^{8}	6×10^{8}	—	—
	Bacitracin 2·0*		8×10^{6}	5×10^{5}	5×10^{6}	1	—
	Streptomycin 20·0		2×10^{6}	3×10^{7}	5×10^{8}	1	—
	Baci. 2·0* + strepto. 2·0		10^{6}	2×10^{3}	10^{3}	1+1/10	Synergism
	Baci. 2·0* + strepto. 1·0		4×10^{6}	2×10^{5}	10^{7}	1+1/20	No synergism
	Baci. 0·5* + strepto. 20·0		10^{4}	5×10^{2}	2×10^{2}	1/4+1	Synergism
	Baci. 0·2* + strepto. 20·0		9×10^{4}	4×10^{6}	7×10^{7}	1/10+1	No synergism
Staph. aureus De	None	10^{7}	3×10^{7}	10^{8}	4×10^{8}	—	—
	Streptomycin 20·0		3×10^{4}	2×10^{7}	3×10^{8}	1	—
	Terramycin 2·0		4×10^{6}	5×10^{5}	9×10^{7}	1	—
	Terra. 2·0 + strepto. 4·0		10^{5}	6×10^{2}	10^{2}	1+1/5	Synergism
	Terra. 2·0 + strepto. 2·0		6×10^{6}	7×10^{4}	8×10^{5}	1+1/10	No synergism
	Terra. 0·5 + strepto. 20·0		2×10^{4}	10^{2}	$<10^{1}$	1/4+1	Synergism
	Terra. 0·2 + strepto. 20·0		10^{5}	4×10^{8}	$>10^{8}$	1/10+1	No synergism
K. pneumoniae AD	None	9×10^{6}	4×10^{7}	3×10^{8}	8×10^{8}	—	—
	Penicillin 0·6		3×10^{6}	7×10^{6}	5×10^{6}	1	—
	Streptomycin 5·0		4×10^{3}	3×10^{7}	6×10^{7}	1	—
	Pen. 0·6 + strepto. 5·0		4×10^{2}	10^{2}	$<10^{1}$	1+1	Synergism
	Pen. 0·6 + strepto. 1·0		7×10^{7}	5×10^{6}	7×10^{6}	1+1/5	No synergism
	Pen. 0·15 + strepto. 5·0		10^{6}	8×10^{5}	2×10^{6}	1/4+1	No synergism

* In units/ml.

The picture was different when the synergism of penicillin with streptomycin against *Strep. faecalis* was explored. This system exhibited several peculiarities. Penicillin showed an optimum zone phenomenon in its action on *Strep. faecalis* (Eagle, 1951). The bactericidal rate was greatest near concentrations of 6 μg./ml., and diminished with both larger and smaller concentrations of drug. Synergistic effects were conveniently tested at this optimum concentration of penicillin because the bactericidal rate was increased beyond the maximum obtainable with *any* concentration of the single drugs. *Strep. faecalis* 16 was very resistant to streptomycin alone; the M.E.D. was 300 μg. streptomycin/ml., no significant bactericidal action was observed with less than 500 μg./ml., and concentrations below 200 μg./ml. had no observable action whatever. However, striking synergism occurred with 0·3 μg. streptomycin/ml. (1/1000 M.E.D.) added to 6 μg. penicillin/ml. (Table 3, Fig. 1). Penicillin concentration could not be decreased correspondingly. With 1 M.E.D. of streptomycin, 1/10 of the optimal penicillin dose failed to give synergism. When the dose of penicillin was increased 25-fold over the optimum, and consequently the bactericidal rate diminished, synergism with streptomycin was likewise reduced (Table 3).

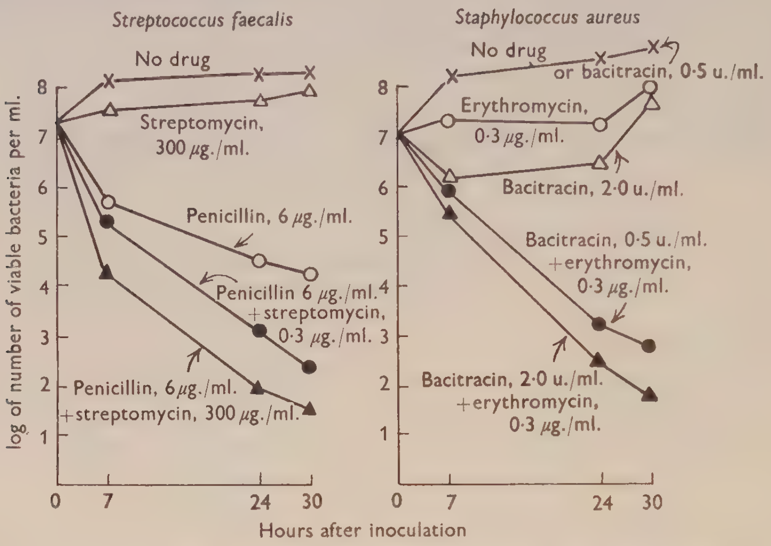


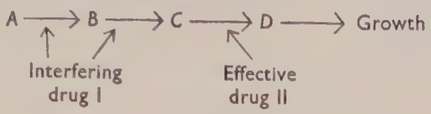
Fig. 1. Quantitative relationships in antibiotic synergism. Left: synergism of similar magnitude results from the combination of streptomycin in either a barely bacteriostatic concentration (300 μg./ml.) or in 1/1000 of that amount (0·3 μg./ml.) with the optimal concentration of penicillin (6 μg./ml.). Right: synergism of similar magnitude results from the combination of bacitracin in either an inhibitory concentration (2 units/ml.) or in an ineffective amount (0·5 units/ml.) with a bacteriostatic concentration of erythromycin (0·3 μg./ml.).

Table 3. *Synergistic effects of varying proportions of penicillin and streptomycin acting on* Streptococcus faecalis *16*

Antibiotics (μg./ml.)	Period after inoculation (hr.) 0	7	24	30	Minimum effective dose of single drugs	Combined effect
	Number of viable bacteria/ml.					
None	4×10^7	3×10^8	4×10^8	4×10^8	—	—
Streptomycin 300·0		8×10^6	5×10^6	10^8	1	—
Penicillin 2·0		5×10^6	6×10^5	2×10^5	1	—
Pen. 0·6 + strepto. 300·0		10^6	6×10^6	3×10^7	3/5 + 1	No synergism
Pen. 2·0 + strepto. 300·0		4×10^5	5×10^2	10^2	1 + 1	Synergism
Pen. 2·0 + strepto. 3·0		6×10^4	5×10^4	10^4	1 + 1/100	Synergism
Pen. 2·0 + strepto. 0·3		10^6	7×10^5	2×10^5	1 + 1/1,000	No synergism
Penicillin 6·0		7×10^5	4×10^4	2×10^4	3	—
Pen. 6·0 + strepto. 3·0		5×10^4	8×10^2	3×10^2	3 + 1/100	Synergism
Pen. 6·0 + strepto. 0·3		3×10^5	10^3	5×10^2	3 + 1/1,000	Synergism
Pen. 6·0 + strepto. 0·1		8×10^5	4×10^3	7×10^4	3 + 3/10,000	No synergism
Pen. 150·0		2×10^6	10^5	7×10^4	75	—
Pen. 150·0 + strepto. 15·0		6×10^4	6×10^2	9×10^1	75 + 1/20	Synergism
Pen. 150·0 + strepto. 1·0		3×10^6	2×10^5	4×10^4	75 + 3/1,000	No synergism

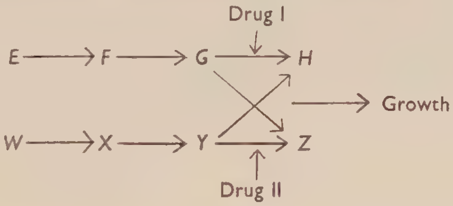
DISCUSSION

If it be assumed that antibiotic action is based on a metabolic block in biosynthetic pathways, then antibiotic antagonism may be pictured as follows, using the diagram proposed by Eagle (1951):



A drug (I) may interfere with the maximal effect of another drug (II) by partially blocking some metabolic process ($A \rightarrow B \rightarrow C$) which is necessary for the optimal action of the 'effective' drug II. If interfering quantities of drug I are bacteriostatic then the slowing of multiplication might account for diminished effectiveness of drug II, e.g. penicillin which is most lethal for rapidly multiplying organisms. Thus bacteriostatic agents like chloramphenicol, chlortetracycline or oxytetracycline might be expected to antagonize penicillin, as indeed they do under certain circumstances. Reasons have been presented elsewhere, however, why bacteriostasis as such probably does not account for the phenomenon of antibiotic antagonism (Jawetz & Gunnison, 1953). In all cases of antagonism studied, the interfering drug has some antimicrobial effect of its own. Thus it is not surprising that the biologically inactive antibiotic degradation products tested in the present study lacked interfering ability.

Antibiotic synergism may be schematically presented in the following way:



A micro-organism may be capable of bypassing a metabolic block produced by drug I in one pathway ($E \rightarrow F \rightarrow G \rightarrow H$); drug II then may act by blocking an alternate metabolic pathway ($W \rightarrow X \rightarrow Y \rightarrow Z$). In the absence of inhibitory drugs, the micro-organism may utilize the biosynthetic processes $E \rightarrow F \rightarrow G \rightarrow H$ with high efficiency, but $W \rightarrow X \rightarrow Y \rightarrow Z$ with low efficiency. Thus a drug blocking $E \rightarrow F \rightarrow G \rightarrow H$ would appear to be inhibitory when acting alone, but a drug acting on $W \rightarrow X \rightarrow Y \rightarrow Z$ may not manifest its effect when present alone. Activity of drug II would be made manifest only by the presence of another inhibitory agent. It seemed plausible, therefore, that chemical substances which were devoid of independent antimicrobial activity might likewise participate in such combined action. The greatest opportunity for such a demonstration appeared to lie with compounds closely related chemically to active antibiotics. The results reported in this paper suggest that synergistic effects occur only with substances which possess independent antimicrobial action in *some* concentration. The ratio of the amount which is capable of participating in synergism to that which exhibits independent antimicrobial activity varies with each drug and each test micro-organism, but is usually from 1/20 to 1/3 of the smallest quantity which gives slight, temporary inhibition of growth. Streptomycin presents a unique exception, because as little as 1/1000 of the minimal effective concentration can sometimes participate in synergism, as Chabbert (1953) has also reported. It appears that streptomycin may affect a biosynthetic process which requires very high concentrations of the drug for complete block yet can be partially blocked by very small concentrations when another drug acts on the organism simultaneously. These results perhaps explain the frequency with which streptomycin participates in synergistic effects even against micro-organisms which appear to be resistant to high concentrations of the drug.

We are grateful to Dr O. Wintersteiner (Squibb Institute for Medical Research, New Brunswick, N.J., U.S.A.) and to Dr D. Rowley (Wright-Fleming Institute, St Mary's Hospital, London) for furnishing the degradation products of penicillin and streptomycin and for much helpful advice. Dr G. Rieveschl (Parke, Davis and Co., Detroit, Michigan) kindly supplied chloramphenicol and the biologically inactive isomer from the D-base. This work was supported in part by a grant from the U.S. Public Health Service (E-214) and from the Research Committee of the University of California School of Medicine.

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(*Received* 7 *August* 1953)

CZEKALOWSKI, J. W., McLEOD, J. W. & RODICAN, J. (1954). *J. gen. Microbiol.*, **10**, 199–208.

Observations on the Nutrition of Leptospira based on the Development of Dinger's Phenomenon

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SUMMARY: The speed of development of narrow zones of opacity due to massed growth of leptospira a few millimetres below the surface in cultures in soft agar, a phenomenon first fully described by Dinger (1932), has been used as a criterion of the nutritional value of media for certain pathogenic leptospira. In applying this test to assess the value of the addition of peptone to a basal medium of 10% (v/v) rabbit serum in diluted meat extract, the impression was formed that peptones contained substances which promoted growth and others which were inhibitory. Witte's peptone was the most constantly favourable of those which were tested. Tryptic digest broth (Hartley's) gave results at least as good. Inhibitory effects with peptone may develop on autoclaving, especially at high pH values, or on leaving sterilized solutions to stand for considerable periods at room temperature. The value of haemoglobin (as laked blood) may depend on its catalase content since Czekalowski, McLeod & Rodican (1953) showed that leptospira did not produce this enzyme. The range of pH suitable for the development of cultures is narrow with pH 7·6 as optimum; but the leptospira are more tolerant to divergences on the alkaline side in the media at the time of inoculation.

Dinger (1932) described the development in cultures of leptospira, in deep tubes of soft agar, of dense narrow rings of growth a few millimetres below the surface which were very sharply demarcated on the under side but less so above. These resembled opaque disks inserted in a cylinder of medium which was otherwise relatively transparent; although a photograph of something very similar had been published by Fletcher earlier (1928) the phenomenon has till quite recently received little attention. It was investigated afresh by Lawrence (1951) and by Czekalowski, McLeod & Rodican (1953). Since this appearance is very characteristic, is only observed when the leptospira are growing well and can be accepted as evidence that the culture is in fact one of leptospira, we considered that it might be used as a criterion of the value of various media for the growth of these micro-organisms.

The outstanding early work on the culture of leptospira was that of Noguchi (1918); but in recent years the media mainly used have been (*a*) Korthof's (1928) medium which is stated by Wiesmann (1952) to be the most widely adopted at the present time, (*b*) Vervoort's (1923) medium or modifications of it, and (*c*) Fletcher's (1928) medium. The basis of the first two is a dilute solution of Witte's peptone (*c.* 0·1%), but whereas Korthof dissolved the peptone in a balanced solution of salts to obtain a pH of 7·2–7·4 Vervoort used dilute phosphoric acid in order to obtain a pH of 6·6 which was corrected to 7·1 on addition of 10% (v/v) of serum. Fletcher's medium differs in that the salt balance and phosphate content depend on the dilution of nutrient agar; the peptone used was not specified. In Britain, amongst those who have worked most with leptospira in recent years, Broom (personal communication) has used a modified Korthof medium consisting of 7 % (v/v) of serum in Locke's solution with a little added haemoglobin; Davidson, Campbell, Rae & Smith (1934) used a medium which they described as that of the Dutch workers. Stuart (1946), who has made a very thorough study of the nutrition of leptospira, introduced a new medium which he has compared with those of Korthof and Schüffner.

One respect in which all media recommended for the growth of leptospira are alike is that their content of commonly used organic nitrogenous constituents is low. The only account of detailed experimental work on which this practice is based is that of Noguchi (1918). He stated that the organisms cannot be grown in any of the usual bacteriological media and that in serum + Ringer solution media, which are otherwise suitable, even 1·5 % of peptone will cause marked suppression of growth. The modern media used by continental workers contain only 0·1 % peptone and it is stressed that this should be Witte's. Broom uses neither peptone nor meat extract; Stuart (1943, 1946) recommended low concentrations of asparagine and NH_4Cl as sources of nitrogen. Stuart found his medium superior to those of the Dutch in ability to yield abundant growths of the largest proportion of all strains of leptospira tested, although he admitted that the Dutch media had the advantage of being suitable with a wider range of rabbit sera.

There is a consensus of opinion that serum is essential, and that of those readily available rabbit is best at a concentration of about 10 % (v/v). Some rabbit sera are inhibitory, however, and Stuart (1943) found fifteen such out of 104 tested. They did not fail owing to deficiency, and the inhibitory effect was not due to adverse pH values nor to the presence of antibodies; the inhibition disappeared on heating to 80° and was partly removed by ether extraction. Opinion is divided about the value of haemoglobin; whereas it is recommended by some experienced workers as a useful addition to media, Wiesmann (1952), using Korthof's medium prepared with fresh rabbit serum (obtained under aseptic conditions and not filtered), did not observe any advantage from adding it. Another aspect in which there is diversity of practice in preparing media for leptospira is in the pH value of the basal medium in which serum is incorporated.

In the Dutch papers pH values ranging from 6·6 to 7·4 are recommended (Vervoort, 1923; Korthof, 1928; Dinger, 1932; Walch-Sorgdrager, Schüffner & Bohlander, 1938; van Thiel, 1948). Walch-Sorgdrager *et al.* (1938) met Zuelzer's (1936) criticism that agglutination results may be adversely affected in slightly acid media with the statement that basal media of pH 7·1 as used by them are invariably brought to pH 7·5 by the addition of the fresh rabbit serum; this they consider to be an optimal pH value. Stuart (1943), however, pointed out that rabbit sera of pH 7·0 may be met occasionally and that samples of medium complete with serum may with time fall below pH 7·0. For this reason he recommended the addition of a trace of phenol red as indicator and used buffer at pH 7·6 in making up his basal medium.

It seemed to us that in view of these varying recommendations it would be useful to check the value of certain ingredients and properties of media used in cultivating leptospira, taking speed of development of the Dinger disk as an easily observed and reliable criterion of a good medium. Our investigations were chiefly related to the value of various protein digest products and to the influence of the pH value of the medium on the initiation and persistence of growth.

EXPERIMENTAL

Organisms. The strain Wijnberg of *Leptospira icterohaemorrhagiae* and strain Aldgate of *L. canicola* were used throughout.

Effect of different solutions and methods of preparation on leptospiral growth

Comparison of Witte and Oxo peptones. In view of the complete failure of all media made up with one batch of Oxo peptone and the uniformly successful results obtained with another we began by comparing a third batch (which was not inhibitory in the way that the second had been) with an old sample of Witte peptone from laboratory stock, since it appears to be generally accepted that Witte peptone is that most suited to the growth of leptospira. In all these experiments the basal medium was extract of fresh meat (beef) 500 g./1000 ml. diluted 1/8 or 1/10 with water and sterilized by filtration through a Maassen or Chamberland candle after addition of 10 % (v/v) of rabbit or other suitable serum. This medium was heated for 30 min. or more at 56° and, after addition of the substance under investigation, three parts of the fluid medium so obtained were added to one part of 4 % (w/v) agar so as to give a final agar concentration of 1 %. The completed medium (6·0 ml.) was held for 10 min. or more at 39° in a water bath, inoculated with 0·25 ml. of a liquid culture of leptospira, thoroughly mixed with a large pipette and allowed to set. It was incubated at 30°. The peptone solutions used in the first experiment were made up at 4 % (w/v), brought to pH 7·8 and sterilized in the autoclave at 10 lb./sq.in. for 20 min. The results obtained with varying concentrations of the two peptone solutions are recorded in Tables 1 and 2. From these the superiority of Witte is very clear, especially with the standard Wijnberg strain of *L. icterohaemorrhagiae.* Within the range of concentrations used the effects of Witte peptone were wholly favourable, whereas the optimum in the middle of the range with this particular batch of Oxo peptone suggested the presence of both favouring and inhibitory materials. The *L. canicola* strain Aldgate was much less sensitive to inhibition by the Oxo peptone, grew more rapidly in all media, but like the Wijnberg strain came up first in the tube with the highest concentration of Witte peptone. This experiment differed from most of those made subsequently in that the medium was made up with a human serum which had been found suitable for culture of leptospira.

Other peptones. In a preliminary experiment in which the methods of preparation of peptone and media were similar to those just described the peptones investigated were those of Armour, Evans, Oxo, Parke Davis, Witte, Allen & Hanbury's Eupeptone No. 2 and the Difco products, Bacto-, Neo- and Proteose-peptones. Good results were obtained with the peptones of Evans and of Parke Davis; fairly good with Difco-proteose and Oxo peptones; poor with Armour and Witte peptones. Eupeptone and the Difco Neo- and Bacto-peptones failed to promote growth. A peculiar observation, however, was that on retesting these solutions after they had stood at room temperature for 14 days several of these results were reversed. These observations were also peculiar in that it was the only experiment of many in which Witte peptone proved less good than other peptones tested simultaneously. Following these results, Eupeptone, Evans, Oxo and Witte peptones were selected for further study.

Table 1. *Growth of* Leptospira icterohaemorrhagiae, *strain Wijnberg as affected by peptone concentration*

The media were obtained by adding the appropriate volume of a 4 % (w/v) solution of peptone (pH 7·8) which had been autoclaved for 20 min. at 10 lb./sq.in. to a filtrate of 10 % (v/v) serum in diluted meat extract and incorporating clear water agar so as to give an agar concentration of about 1 % in the completed medium.

		Period of growth (days)					
		1–3	5	7	9	12	21
Peptone	Concn. (%)	Degree of growth (mm.)					
Witte	1·5	—	● 8	● 10	● 10	● 10	● ● } 13 ●
	0·75	—	—	● 9	● 9	● 10	● } 12 ●
	0·375	—	—	● 11	● 11	● 12	● } 12 ●
	0·19	—	—	● 12	● 12	● 12	● } 13 ●
	0·075	—	—	● 11	● 11	● 12	○ } 13 ●
	0·0375	—	—	○ 14	● 14	● 13 ● 14	● 12
Oxo	1·5	—	—	—	—	—	—
	0·75	—	—	—	—	—	—
	0·375	—	—	—	—	—	● 9
	0·19	—	—	—	—	○ 10–11	● 13 ● 15
	0·075	—	—	○ 10	● 10	● 10–11	● 13 ● 15
	0·0375	—	—	—	—	—	—

○ = faint indication of disk; ● = frank disk; — = no disk.

Figures indicate approximate distance below surface at which disk appears, e.g. ●●● } 13 means three disks near together about 13 mm. below surface.

Four methods of preparation of peptone solutions were compared: (*a*) a 1 % (w/v) solution was brought to pH 7·3 and autoclaved at 10 lb./sq.in.; (*b*) a 4 % (w/v) solution was brought to pH 8·0 and autoclaved at 10 lb./sq.in.; (*c*) and (*d*) as (*a*) and (*b*) but sterilized by filtration. The following results were obtained.

Table 2. *Growth of* Leptospira canicola, *strain Aldgate as affected by peptone concentration*

Peptone	Concn. (%)	Period of growth (days)					
		1–2	3	5	7	14	21
		Degree of growth (mm.)					
Witte	1·5	—	○ 8	● 8	● 8	● 10 ● 11	● ● } 13 ●
	0·75	—	—	● 8	● 8	● } 10 ●	○ ○ } 11 ○
	0·375	—	—	● 8	● 8	● 10 ● 11	● ● } 12 ●
	0·19	—	—	—	● 9	● 9	● } 11 ●
	0·075	—	—	—	● 9	● 9	● } 11 ●
	0·0375	—	—	—	● 8	● 9	● } 12 ●
Oxo	1·5	—	—	—	○ 10	● 9	● 9
	0·75	—	—	○ 8	● 9	● 8	● ● } 10 ●
	0·375	—	—	—	○ 10	● 8	● ● } 11 ●
	0·19	—	—	—	○ 10	● 10	● ● } 12 ●
	0·075	—	—	—	—	● 8	● } 11 ●
	0·0375	—	—	—	—	● 9	● } 12 ●

Signs in this and in succeeding tables have the same significance as in Table 1.

(*a*) With a peptone concentration 0·5 % (w/v) in the completed medium all tubes containing peptone showed disk formation more rapidly than the culture in the control tube of basal medium when the *L. canicola* strain Aldgate was inoculated. With the strain Wijnberg, however, there was no evidence of growth in the tube containing Evans peptone, even up to 3 weeks incubation, although a well-defined disk had appeared in the control. In the media containing Witte peptone disks appeared 2–3 days before those in the media containing other peptones and at least a week before those in the control.

(*b*) Using the same final concentration of peptone as in (*a*) but a solution of peptone autoclaved at pH 8·0, no evidence of promotion of growth in the presence of peptone was obtained. All peptones except Witte appeared to be more or less inhibitory, and the result with Witte was no better than that obtained with the basal medium control.

(*c*) Using solutions as in (*a*), but sterilized by filtration, all peptones were favourable with the strain Wijnberg, Witte being the best. With the strain

Aldgate the Witte peptone again proved to be the best and superior to the control, whereas the three other peptones gave more retarded growth than in the control.

(*d*) Using solutions prepared as in (*b*), but in a final concentration of 0·12 % and sterilized by filtration, no inhibitory results were obtained. Witte was still the best, and Evans and Eupeptone showed accelerated growth as compared with that in the control.

To sum up, these experiments suggest that filtered peptone solutions are better than autoclaved solutions and inhibitory effects are most likely to develop in slightly alkaline solutions of autoclaved peptone. In view of these findings an experiment on the effect of sterilizing peptone solutions by heat at different pH values was made.

The pH *values of cultures of leptospira and their correlation with profusion of growth*

Basal media of pH values as low as 6·6–7·2 have been recommended for the growth of leptospira and this has left the impression that these micro-organisms grow best at pH values slightly lower than those most suitable to the majority of pathogenic bacteria. On account of the importance of this point, and its significance in our experimental work, we examined it afresh, measuring pH values with a glass electrode and a Cambridge pH meter. The only account of observations on the electrometric measurements of pH values of media suitable for leptospiral culture of which we are aware is that of Walch-Sorgdrager *et al.* (1938). They concluded that it was possible to get growth in first subcultures in media ranging in pH values from 5·8 to 8·5, but for repeated subculture pH 7·5 appeared to be the optimum.

Results. Parallel cultures of leptospira were made in a series of fluid media at graduated pH values and in a similar series solidified by agar buffered at pH 7·3. The liquid cultures were observed daily with the microscope (dark-ground illumination) for number and motility of leptospira and the solid cultures for the development of Dinger disks. The results are recorded in Tables 3 and 4. The enumeration of leptospira in liquid media suggested that initiation of growth was most rapid at pH 7·5 but that initial pH values of 7·9 and 8·2 were at least as satisfactory for the ultimate development of well-grown cultures. Values below pH 7·2 or above pH 8·2 delayed initiation of growth and full development of cultures. There was no evidence that the growth of the leptospira influenced the reaction of cultures, but in the liquid there was a marked tendency for the more alkaline media spontaneously to become gradually more acid. Within a week they all fell within the range pH 7·5–7·8 in this experiment, although in another and similar one the acid drift was less marked and free growth appeared to have developed even at pH 8·0. The general indication is of narrow range of suitable pH values, probably not extending beyond 7·2–8·0.

Determinations of pH values in solid media with glass electrodes presented difficulties. The procedure we adopted was to add 25 % (v/v) of 4 % (w/v) water agar buffered at pH 7·3 with 0·01M-phosphate to a series of samples of liquid media adjusted to the desired pH values. Some of these were put up in duplicate, and these were used to determine the shift of pH consequent on the incorporation of the agar. Samples of liquid media containing Witte peptone and Eupeptone respectively were adjusted to pH 8·3 by means of the glass electrode, and after incorporation of the agar were poured into the electrode chamber and allowed to set over the glass bulb. A reading was taken as soon as the solidified medium had reached room temperature; duplicate measurements showed pH of 7·7. It appeared that pH values of the liquid medium which were one pH unit or more alkaline to the pH value of the buffered agar (pH 7·3) changed to a pH about 0·5 unit towards the acid side when the agar was added in the quantity used in our experiments. In Table 4 are recorded both the reaction of the liquid medium to which the water agar was added and the probable reaction of the completed solid medium, computed on this basis. The observations on growth in solid media at different pH values recorded in Table 4 indicate pH 7·4 as an optimum with a marked check on growth below 7·2 and above 8·0. Other similar experiments were carried out and included some with media prepared with Eupeptone and inoculated with *L. canicola* Aldgate. The results were all very similar, but some of them indicated a rather greater tolerance of alkaline reactions than shown in Table 4. In all, the indication seems to be that there is a rather narrow pH range for optimal growth, a rather marked sensitivity to acid reactions and more tolerance for media of which the initial reaction was too alkaline rather than too acid.

Table 3. *Effect of* pH *on development of leptospira in a favourable fluid medium and its variation with growth of the leptospira*

Initial pH of medium	Number of leptospira per field (dark background) on successive days						Observation of pH during culture
	2nd	3rd	4th	6th	8th	26th	
7·2	1, active	5, active	15, active	30–50, active	30, fairly active	20–30, long and sluggish	
7·5	2, active	30, active	> 50, active	50–70, active	50–100, active	50–60, long mod. active	5th day: 7·56 (7·64)*
7·94	2, active	10, active	15, active	100, very active	> 100, active	> 100, mostly very active	7·8 (7·84)*
8·2	4, active	7, active	15, active	50–60	> 100, active	50–60, rather sluggish	8th day: 7·5 (7·5)*
8·6	4, active	5, some sluggish	30, active	100	25, active	10–20, rather sluggish	7·7 (7·7)*
8·8	2, sluggish	4, some sluggish	5, sluggish	20–40	5–20, active	70–100, mostly sluggish	

Strain: L. Wijnberg. *Medium*: as for Table 4 without agar.

* Figures in brackets indicate the drift of pH level in uninoculated control samples of media of the same initial pH.

Effect on the nutritive quality of peptone of the pH *value at the time of autoclaving*

The peptone solutions (2 %, w/v) were divided into seven portions and by addition of acid or alkali these were adjusted to a range of pH values between

Table 4. *Development of Dinger's disks in cultures of varying initial* pH *at intervals of days*

pH of fluid	Computed pH of solid medium	4th day	5th day	6th day	7th day	8th day	9th day	16th day	23rd day
7·2	7·2	—	—	—	—	○	●	● ●	● ●
7·5	7·3	—	—	●	●	● ●	● ●	● ● ●	● ●
7·94	7·5	—	●	●	● ●	●	● ●	● ● ●	● ●
8·2	7·7	—	—	○	●	●	● ●	● ●	● ●
8·6	8·0	—	—	—	●	●	● ●	● ●	● ●
8·8	8·2	—	—	—	—	—	—	●	●
	8·6*	—	—	—	—	—	—	—	—

Strain: L. Wijnberg.

Medium: pooled rabbit serum 10 % (v/v)
Witte peptone 0·1 % (w/v)
10 % meat extract in aq. dist. 90 % (v/v)
} 3 parts to 1 part 4 % (w/v) of water agar.

* Actual pH determined after addition of alkali.

4 and 8·9 before autoclaving at 10 lb./sq.in. for 20 min. The reaction of each was then adjusted and after the addition of the other ingredients of the liquid media all were found to be within the pH range 7·3–7·5, with one exception. This was the sample of Witte peptone which had been autoclaved at pH 4 and had only been brought back to pH 6·9. It was assumed, in view of the results above, that the further addition of agar would leave all media so near the optimal reaction for growth that the conclusions with regard to the effect of autoclaving peptone at different pH values were likely to be valid. The concentration of peptone in the completed media was 0·5 % (w/v). The media were inoculated as before with the strains Wijnberg and Aldgate. With Eupeptone the pH range actually obtained was from 4·34 to 8·9. There was complete inhibition of both strains by the peptone solution autoclaved at pH 8·9. All samples autoclaved at pH values between 7·9 and 4·34 showed well-developed disks after 3, 4 or 5 days of incubation. With Witte peptone the range of pH values obtained was from 4 to 8·74 at time of autoclaving. No inhibition of growth was observed. There was slight retardation of growth in a few cultures, and slight acceleration in some others.

Effect of different concentrations of meat extract

The leptospira tolerated considerably higher concentrations of meat extract than those commonly used in media such as Fletcher's, but they did not tolerate those used in everyday bacteriological work. This inhibition at high concentrations of meat extract was found to be diminished in the presence of Witte peptone but increased with the Oxo product.

Tryptic digest broth. Hartley's tryptic digest broth, modified according to Mackie & McCartney (1950), in concentrations of 8–60 % (v/v) was solidified with water agar and compared with the meat extract Witte peptone agar of varying peptone content from 0·16 to 0·4 % (w/v). Both series of media included the usual amount of rabbit serum. In two experiments the media with Hartley broth proved superior; although this cannot be claimed to be significant without further trials we were impressed with the fact that this was the only medium amongst those tested which showed development of a second disk as early as the third day.

DISCUSSION

The results recorded show that most peptones in suitable concentrations will increase the speed of the growth of leptospira in a basal medium of 10 % (v/v) rabbit serum in diluted extract of meat, although the best samples of the basal medium grow the leptospira well without any addition of peptone. Inhibitory effects, however, occur with many peptones, especially when they are at concentrations above 0·1 %. The cause of such inhibitory effects is not clear, but they are more liable to occur when the peptone is autoclaved at pH 7·8 or more. They are rarely met, however, with Witte peptone, and in many experiments better growth was obtained with concentrations of this product much higher than those generally recommended. Some bizarre results were obtained when different peptones were compared immediately after autoclaving and again after standing in solution for a fortnight at room temperature exposed to diffuse daylight. Some were favourable for growth at the outset but inhibitory after standing, while the reverse effect was observed with others.

Czekalowski *et al.* (1953) drew attention to the fact that leptospira are devoid of catalase. On this account they may be particularly sensitive to the traces of H_2O_2 which tend to accumulate in solutions containing organic matter when allowed to stand exposed to light (see Bedford, 1927). This may explain the advantage often claimed from adding haemoglobin to media for growing leptospira. When the haemoglobin is added as laked blood (the usual practice), it is of course a material rich in catalase. Wiesmann (1952) stated that he found no advantage in adding haemoglobin to his media, but since he insisted that the rabbit serum used must be fresh, this may only mean that there is no advantage in adding catalase to a medium which already contains it. Our observations are in line with those of Walch-Sorgdrager *et al.* (1938) in finding pH 7·5–7·6 as the optimum for leptospiral growth, which might be expected with a pathogenic organism which invades animal blood and tissues. Our observations suggest, however, that sensitivity to variations from this pH value are more marked on the acid than on the alkaline side and that a basal medium of pH 7·6–7·8 is probably safer than one of 6·8–7·2, as often recommended. It is interesting in this connexion that Thiry & Meirhaeghe (1932), in a study of the optimum pH for growth of leptospira in Vervoort medium no. 2, found this to range from pH 7·8 to 9·0 for six strains of *L. icterohaemorrhagiae* and from pH 6·6 to 7·2 for two leptospira strains from water. Three of their *L. icterohaemorrhagiae* strains failed at pH 7·0. They do not state, however, how their pH values were determined.

We are much indebted to Mr G. Eaves for help in translating Dinger's paper from the Dutch, to Mr T. Varley for much painstaking work in the preparation of media and to the Medical Research Council for a grant.

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The Rate of Formation of Hyaluronidase, Coagulase and Total Extracellular Protein by Strains of *Staphylococcus aureus*

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SUMMARY: Hyaluronidase is formed as a constitutive enzyme by *Staphylococcus aureus* growing in either broth or casein hydrolysate media. The lag in the appearance of hyaluronidase activity after inoculation of a culture is longer than the lag in growth. A change in the rate of formation of hyaluronidase therefore occurs after exponential growth has been established. Activity per unit volume of culture supernatant increases exponentially faster after this initial lag than the mass of bacteria; it stops increasing abruptly sooner than growth. Coagulase appears, after inoculation, without lag additional to that in growth but increases at a slower rate than growth until it too ceases to increase before growth stops. Evidence is presented to show that changes in enzymic activity of the culture represent enzyme formation and not secondary changes in formed protein or secretion phenomena. The changes in rate of enzyme formation are connected with processes controlling enzymic specificity of the proteins rather than with general processes involved in manufacturing extracellular protein.

It was shown some years ago that whereas *Clostridium welchii* and streptococci produce more hyaluronidase when casein hydrolysate or peptone media are supplemented by hyaluronate, staphylococcal enzyme formation is unaffected by the addition of hyaluronate (Rogers, 1945). Thus hyaluronidase appears to be an adaptive or induced enzyme in the former species but a constitutive one in the latter. Further analysis of the course of production of hyaluronidase by staphylococci has been undertaken in order to investigate the mechanism of constitutive enzyme formation.

Little information exists on the course of enzyme formation, particularly of extracellular enzymes, under circumstances which preclude adaptive response to substrates in the growth medium; also, in most of the cultures studied the organisms have ceased to multiply exponentially. Attention in the present work has been concentrated on changes in the rate of hyaluronidase, coagulase, and protein formation which occur during the logarithmic phase of growth. A steady state can never be established in an ordinary culture, but changes in enzyme formation which occur during a period of exponential growth must be due to some change in the culture which alters enzyme formation without affecting growth. For example, the cells may accumulate some substance necessary for hyaluronidase or coagulase formation, or inhibitors may be removed from the medium. In order to be able to correlate the measured hyaluronidase activities with the formation of the enzyme an attempt has been made to exclude activation of any pro-hyaluronidase or inactivation of formed enzyme. The amount of cell-bound hyaluronidase has also been examined because, from the results obtained, it seemed possible that the cells might be accumulating hyaluronidase in the early stages of growth and releasing it later.

The course of coagulase formation has been chosen as a comparative study to that of hyaluronidase, partly because results obtained by Davies (1951) suggest that coagulase may be formed very rapidly under some circumstances once the cells have started to multiply, whereas the present work has shown that in early growth hyaluronidase formation lags.

METHODS

Cultural conditions and strains of organism. Two strains of *Staphylococcus aureus* were used for the detailed work reported here (i.e. 524 and Newman); both were isolated by Dr C. H. Lack. A description of the isolation of variants from 524 has been published elsewhere (Rogers, 1953). Similar results, not given here in detail, have been obtained with two other strains (isolated by Dr R. E. O. Williams) which produced sufficient hyaluronidase and coagulase to allow measurement of both in the same culture. Unless otherwise stated, the organisms were grown in lightly plugged Ehrlenmeyer flasks containing 0·2 of their volume of medium, shaken 88 times per min. with a horizontal traverse of 8 cm. in an incubator maintained at 35 ± 0·5°. The media were warmed to 35° on the morning of the day of the experiment and inoculated with 0·15 ml./100 ml. of an 18 hr. culture of the particular strain to be investigated, grown in Hedley Wright broth buffered with 2·5 % (w/v) sodium glycerophosphate and containing 0·25 % (w/v) glucose. Two media have been used: (*a*) The above-mentioned buffered Hedley Wright broth; (*b*) the supplemented casein hydrolysate medium previously described as medium B (Rogers, 1945). Growth took place exponentially during a considerable proportion of the total growth in both media.

The cultures were usually sampled at half-hourly intervals by withdrawing the required volumes aseptically from the flasks and delivering them into 'lusterlite' centrifuge tubes immersed in a mixture of ice and water. Samples from these tubes were then taken into ice-cold diluting fluid for the measurement of bacterial growth, and the tubes were centrifuged for 15 min. or longer at about 4000 r.p.m. in a centrifuge kept at 0–4°. The supernatants were then removed, a drop of toluene added and they were stored at 0–4° or at −2° for not longer than 24–48 hr.

Estimation of bacterial growth. The mass of bacterial growth was estimated by the opacity of suitable dilutions of the cultures using a Spekker photoelectric colorimeter and an Ilford neutral grey filter H508. The size and grouping of staphylococci change during growth and it was, therefore, necessary to make sure that any change in the relationship between opacity, dry weight and total bacterial nitrogen (used as an approximate estimate of bacterial protein) was not sufficient to interfere with the interpretation of the results. Five samples were taken from a culture of *Staph. aureus* (strain 524) growing in the broth medium, at 4, 6 and 20 hr. after inoculation. Sample 1 was diluted and the opacity of a range of dilutions was measured; duplicate samples 2 and 3 were centrifuged, the cells were washed twice with saline and their total nitrogen content was estimated by a conventional micro-Kjeldahl technique; duplicate samples 4 and 5 were centrifuged, the cells were washed twice with distilled water and transferred to tared weighing bottles and dried to constant weight at 105°.

The nitrogen content as a percentage of the cell dry weight was approximately constant (11·9–12·5 %) for the period over which samples were examined. Fig. 1 shows that for the first two samples the relationship between opacity and bacterial nitrogen was the same within the limits of the methods; but for the sample collected 20 hr. after inoculation the relationship was different. As will be shown later, the most important part of the work was concerned with the period up to 7 or 8 hr. after inoculation. It was, therefore, thought to be satisfactory to use only opacity as a measure of the mass of bacterial growth, and hence of bacterial protein. When overnight cultures were examined, the appropriate curve for the relationship between bacterial N and opacity was used. Results are expressed as mg. bacterial N/ml. culture.

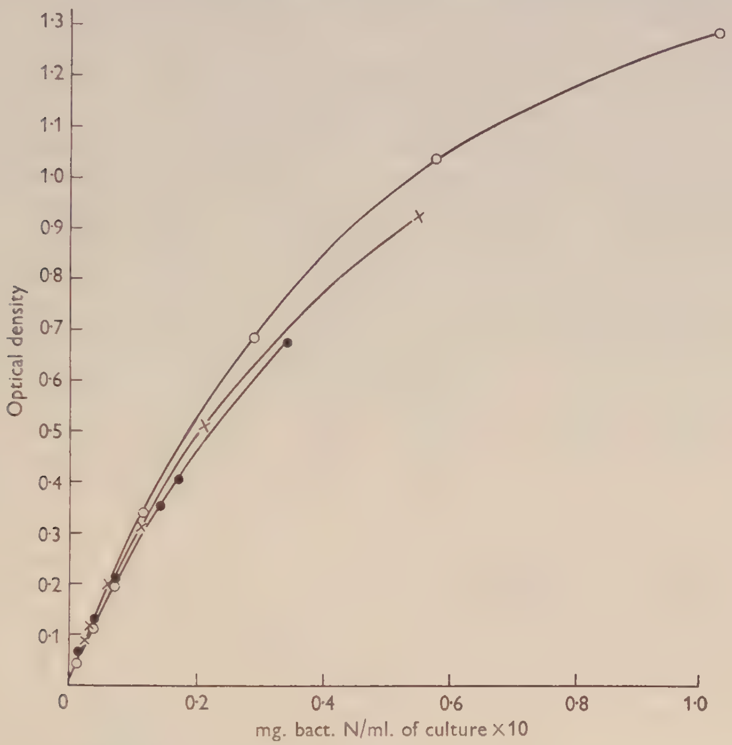

Fig. 1. The relationship between optical opacity and bacterial N (for samples taken at different times from a growing culture of *Staphylococcus aureus* strain 524). –●–, 4 hr. culture; –×–, 6 hr. culture; –○–, 24 hr. culture.

Estimation of hyaluronidase. The Tolksdorf, McCready, McCullagh & Schwenk (1949) modification of the turbidity reduction method was used. Gelatin at a concentration of 0·2 % was present in all the tubes including the standards containing only buffered substrate. The addition of gelatin to the standard tubes as well as the tests is of importance since it has considerable effect upon the turbidity given by the hyaluronate when mixed with the acidified serum reagent. A final concentration of 10^{-4} M-sodium pyrophosphate was also present throughout the tests as an additional guard against heavy metal inactivation of the enzyme (Meyer & Rapport, 1951). Crucial values were always rechecked using three different concentrations of the culture supernatants. The term hyaluronidase must in this paper be taken to mean the enzyme, formed by staphylococci, which breaks down hyaluronate sufficiently to be estimated by the above technique. Results are expressed as turbidity reducing units (T.R.U.) per ml.

Estimation of coagulase. The technique which is commonly employed for estimating the potency of coagulase by finding the dilution of the supernatant which just fails to coagulate either suitable plasma, or a combination of fibrinogen and suitable 'activator', was not sufficiently precise for the purposes of the present investigation. Therefore, coagulase was measured by the time taken by dilutions of the culture supernatants to clot fresh, whole, pooled human plasma under standard conditions. Clotting time was estimated by dropping ballotini glass balls, no. 12 (Chance Bros. Ltd., Birmingham), into a tube containing the plasma-culture supernatant mixture. The tubes were incubated at 37 ± 0·5° in an ordinary glass-sided flocculation bath with a strong light on the side opposite the operator and focused on the tubes. When clotting occurred the brilliantly refractile balls could be seen to stop abruptly in their downward course. A plot of the logarithm of the time taken to clot the plasma against the logarithm of the volume of supernatant used, gave a satisfactory straight line, provided the clotting time was between 1 and 10 min. This double logarithmic relationship was first observed for thrombin clotting by Fischer (1935). An arbitrary unit, the 'clot', was defined as the volume of supernatant which, when adjusted to 0·5 ml. with normal physiological saline, would clot 1·0 ml. of human plasma in 240 sec. at 37° in a tube 0·5 cm. in diameter, agitated by constant tapping.

Estimation of total extracellular protein. The amounts of protein present in supernatants from cultures examined up to 5 or 6 hr. after inoculation were too small to allow the use of conventional methods such as the estimation of trichloroacetic acid insoluble nitrogen. Recourse was, therefore, had to a method designed by Lowry, Rosebrough, Farr & Randall (1951) which combines the biuret and Folin-Ciocalteu phenol estimations using trichloroacetic acid precipitates. This method has been found to be very reproduceable providing great care is taken in centrifuging and handling the very small precipitates obtained. A disadvantage of the method is that the same weights of different proteins give different depths of colour. Therefore, if any considerable change takes place in the types of soluble protein formed during growth of the organisms, the comparative results may be inaccurate. The greatest difference, however, demonstrated by Lowry *et al.* (1951), that between trypsin and gelatin, was a threefold difference in colour. It is thought to be unlikely that the interpretation of the results will be seriously affected since the formation of a high proportion of protein of very low tyrosine content is unlikely.

RESULTS

The course of appearance of extracellular hyaluronidase and coagulase

The potency of hyaluronidase was measured in the supernatants from cultures of the unselected *Staphylococcus aureus* (strain 524) growing in the buffered broth medium. Sampling was started soon after inoculation and continued over most of the growth period. Fig. 2 shows the result of one such experiment, where the logarithms of both the mass of bacterial nitrogen and the enzyme potency in the culture superantant are plotted against time. By extrapolating both of the lines to meet the lines drawn parallel to the time axis through the initial values for the inoculum, two so-called lag periods are obtained. The initial values were calculated from the known dilutions of the culture used for inoculation. Whereas the lag period for growth was only about 1·0 hr., that for the appearance of enzyme was 2·8 hr. Enzyme forma-

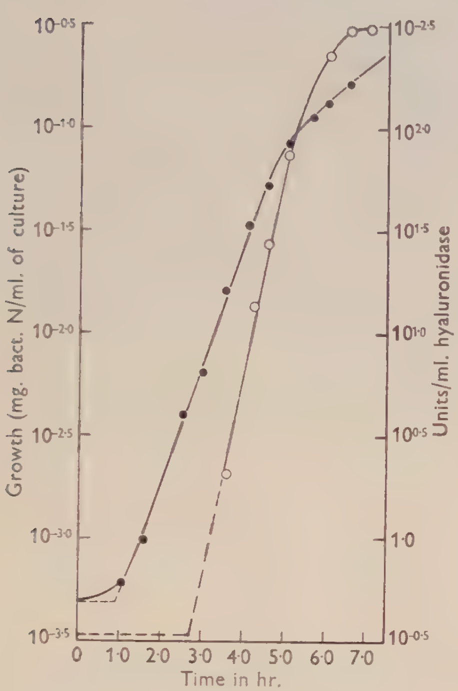

Fig. 2

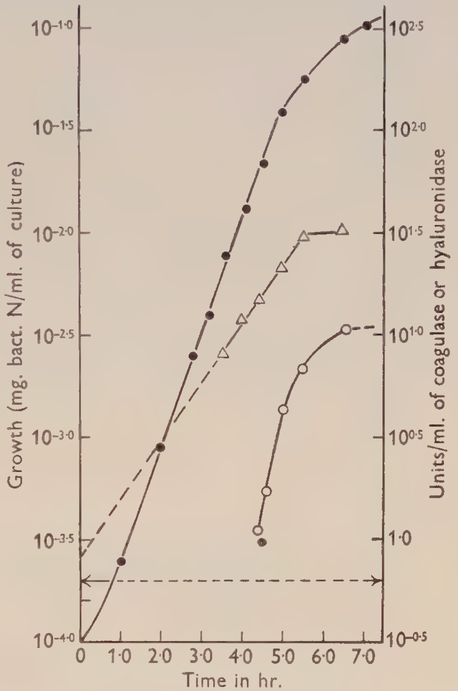

Fig. 3

Fig. 2. The course of growth and hyaluronidase appearance plotted as logarithmic functions relative to the age of the culture, using *Staph. aureus* (strain 524). -●-, bacterial growth as mg. bact. N/ml. of culture; -○-, hyaluronidase as T.R.U./ml. of culture supernatant.

Fig. 3. The course of growth, coagulase and hyaluronidase formation plotted as logarithmic functions relative to time in cultures of *Staph. aureus* (strain Newman). -●-, bacterial growth as mg. bact. N/ml. of culture; -○-, hyaluronidase as T.R.U./ml. of culture supernatant; -△-, coagulase as clots/ml. of culture supernatant. The horizontal broken line indicates the inoculum level of coagulase.

tion thus continues to accelerate after the rate of multiplication of bacterial mass has become constant. The lag period in enzyme formation was rather variable but was always more than twice that for bacterial growth. There seemed to be no correlation between the two. No more enzyme was formed after about 7 hr., whereas growth continued until the mass of bacteria had increased twofold or threefold after 18 hr. incubation. The same type of relationship was obtained using casein hydrolysate medium B, except that the whole pattern of enzyme formation was more variable.

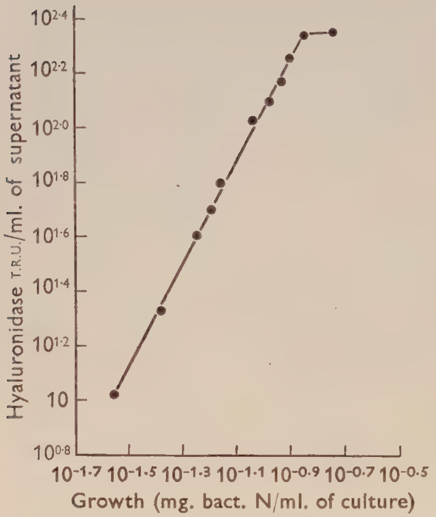

Fig. 4

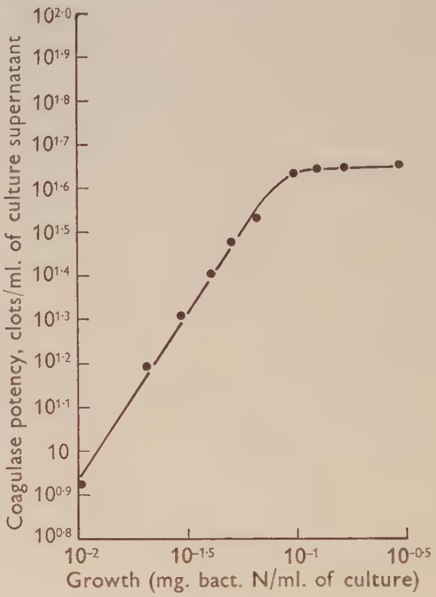

Fig. 5

Fig. 4. The course of appearance of hyaluronidase relative to the amount of bacterial growth plotted as a double logarithmic function. The results represent the mean of five experiments using cultures derived from a single pair of cells of strain 524.

Fig. 5. The double logarithmic function for the appearance of coagulase in cultures of *Staph. aureus* strain Newman. The results represent the mean of two experiments.

When coagulase production by strain 'Newman' was examined a different relationship was obtained. Fig. 3 shows that coagulase appeared rapidly immediately growth began and again ceased before the bacterial mass had stopped increasing. On the same figure are plotted the results for the weak hyaluronidase formed by this strain. The latter enzyme again shows the same type of curve as that obtained for strain 524.

Measurements of the slopes of the logarithmic plots of hyaluronidase and coagulase potency against time showed that the principal difference between the behaviour of the two entities was that after the initial lag hyaluronidase appeared faster than bacterial growth, whereas coagulase appeared more slowly but without initial lag. If this were true, an approximate straight-line relationship should exist for the formation of both when the logarithm of growth is plotted against the logarithm of potency. Figs. 4 and 5 show the results of such plots. The straight line for the production of hyaluronidase is drawn through the results from five different experiments using cultures derived from a single pair of cocci isolated by micro-manipulation (Rogers, 1953). The results for coagulase formation also fall on a straight line but the slope of the line is much less steep.

Thus hyaluronidase appears in the culture more rapidly and coagulase less rapidly than bacterial protein, but there is a lag in the appearance of hyaluronidase during which growth is proceeding at maximum rate. A possible explanation of this lag in hyaluronidase formation could be the progressive activation or inactivation of the enzyme. If, for example, hyaluronidase were unstable in the medium when present in low concentration but were stabilized by an increase in concentration, one might expect the enzymic activity of the culture to display the type of phenomenon described. Alternatively, an inactive soluble pro-enzyme might be formed during early stages of growth and be progressively activated as the bacterial mass increased. To test these two possibilities two types of experiment were done. (1) Samples of supernatant taken from the late production phase of the organisms (strain 524) were diluted in fresh medium so as to give the potency of the hyaluronidase expected if enzyme formation had been in direct relation to growth at the time when growth was equivalent to 0·02 mg. bacterial N/ml. These dilutions were incubated for 4 hr. at 37° and assayed. The greatest degree of inactivation recorded from such experiments was 13 %. Such a loss would be far too small to account for the discrepancy between enzyme actually found (i.e. *c.* 4–5 T.R.U.) and that to be expected (90 T.R.U./ml.). (2) Samples from the early growth phase of the cultures were mixed with those from the middle and end of the enzyme production phase. These mixtures were then incubated at 37° for 90 min., cooled and assayed in the usual way. If inactive pro-enzyme is produced early in the culture and later activated, it should be possible to demonstrate that the mixture has more than the theoretical enzyme potency after incubation. No evidence for activation of early samples by later ones could, however, be obtained. This, of course, does not exclude the possibility of the activation of some pro-enzyme-like substance by the activities of the cells themselves.

Cell associated hyaluronidase and coagulase

The lag period in the formation of hyaluronidase would also be consistent with an accumulation of the enzyme inside the cells followed by release. To test this possibility samples were taken at various stages of growth of the cultures, centrifuged, the cells were washed rapidly once with 1/10 the volume of ice cold 0·1 M-phosphate buffer at pH 7·0 and then resuspended in 1·0 ml. amounts of the same buffer. At first the cells in the suspension were broken by shaking with ballotini glass-balls, no. 12, at 50 cyc./min. (Curran & Evans, 1942). It was subsequently found that the enzyme present in the cells is released on storage of the suspensions in buffer overnight at 0–4°. The latter method was used to estimate the amount of cell-bound enzyme. It is subject to two errors; first, about 10–15 % of the enzyme remains associated with the cells and secondly, an increase in the total potency of the enzyme in the suspension occurs during storage. This increase is very irregular and may be due to traces of medium constituents carried with the cells and not removed during the single washing with phosphate buffer. Washing, however, cannot be made more thorough since a considerable proportion of the enzyme known to be present when cells were immediately broken, was removed. The enzymic activity associated with the cells was small and the errors involved were not likely to affect interpretation of the relation between cell-bound and cell-free enzyme.

It was found that no enzyme could be detected in cell suspensions prepared from 18 hr. cultures; thus the enzymic content of the cells was less than 0·5 T.R.U./mg. bacterial N when these were inoculated into fresh medium. Table 2 shows that as cultures were incubated cell associated enzyme appeared and increased per unit mass of cells until the culture filtrate had reached about a third of the potency it would eventually attain. Thereafter there was a decrease in units of cell-associated hyaluronidase. In order to account for the deficiency in the enzymic potency of the culture at, say, the stage represented by the first sample examined for content of cell-associated enzyme, the cells would have had to contribute *c.* 100 units/ml.; in fact, they could have contributed only 0·8 unit/ml., or 0·8 % of the deficiency. Several experiments of this type were conducted, and suitable controls were included to make sure that enzyme was not destroyed during the process of extraction from the cells. It was concluded that secretion as such does not play a major role in determining the lag in the production of hyaluronidase.

Table 1. *The relation between the potency of hyaluronidase associated with the cells and that in solution in growing cultures of* Staphylococcus aureus *(strain* 524)

Age of culture (hr.)	Mass of growth (mg. bact. N/ml. × 10)	Cell associated enzyme T.R.U./mg. bact. N	T.R.U./ml. culture	Cell free enzyme (T.R.U./ml. culture)
4·0	0·23	33·7	0·78	4
4·75	0·68	53·8	3·6	65
5·2	1·08	61·0	6·6	116
6·0	1·37	24·0	3·2	265
6·8	1·80	3·8	0·68	355
18·0	5·4	<0·5	<1·0	330

Indications were obtained of an approximately constant amount of coagulase associated with the cells of strain 'Newman' throughout growth. Owing to the low potency of this cell-associated coagulase, however, relative to that in the culture supernatant, observations could not be made sufficiently quantitative to be sure.

The production of extracellular protein

It appears from the above results that the formation of hyaluronidase first accelerates and then remains faster than growth, whilst at least one other active substance, coagulase, is formed at a uniformly slower rate than either hyaluronidase or cell dry weight. In order to assess the degree of specificity of these results it is of importance to know the course of production of total extracellular protein. It is possible, for example, that the formation of either hyaluronidase or coagulase is reflecting total protein secretion by the organisms. If so, the course of production of this enzyme presumably would be controlled by factors which also affect the manufacture of a number of other cell proteins. For example, some of the processes involved in the early stages of constructing polypeptide chains might limit the rate of synthesis of several proteins not directly connected with the economy of the cell, but would not necessarily control the enzymic specificity of these proteins.

These experiments were conducted by the same techniques using strain 524 as those already described but in all the experiments medium B had to be used. The amount of trichloroacetic acid precipitable material present in the Hedley Wright medium (A) was far too great to allow the estimation of the very small amounts of protein secreted by the organism. It has, however, been pointed out that the course of enzyme production conforms to the same pattern when the organisms are growing in the casein hydrolysate medium. Two of the variants previously described (Rogers, 1953) were used for this work; one produced very little, the other very potent hyaluronidase.

Table 2. *The formation of hyaluronidase and total extracellular protein by cultures of* Staphylococcus aureus (*strain* 524) *growing in casein hydrolysate medium* (*Medium B*)

(I = less active variant; II = more active variant.)

Time		Growth (G) (mg. Bact. N/ml. × 10)		Hyaluronidase (H) (T.R.U./ml.)		Protein (P) (μg./ml.)		Ratio: H/G		Ratio: P/G	
hr.	min.	I	II	I	II	I	II	I	II	I	II
0	0	0·002	0·004	—	—	—	—	—	—	—	—
1	0	0·003	0·005	—	—	—	—	—	—	—	—
2	0	0·006	0·012	—	—	—	—	—	—	—	—
4	0	0·04	—	—	—	—	—	—	—	—	—
5	0	0·168	0·190	—	—	—	—	—	—	—	—
5	20	0·294	0·218	<0·5	<0·5	—	—	—	—	—	—
5	50	0·350	0·427	<0·5	2·6	—	7·0	—	6·1	—	16
6	20	0·650	0·661	2·1	7·2	8	11	3·2	11	12	17
6	50	1·16	1·03	7·7	37	17	29	6·5	36	15	28
7	20	1·60	1·55	17	63	29	37	11	41	18	24
7	50	2·10	2·19	24	95	40	63	11	44	19	29
8	20	2·27	2·34	33	105	59	79	14	45	26·0	34
9	0	—	2·60	—	134	—	92	—	51	—	34
24	0	4·80	4·82	22·3	132	163	346	4·6	27	34·0	71

Table 2 shows the results obtained. It will be seen that the course of trichloroacetic acid precipitable protein formation is not identical with that of the hyaluronidase over the range of growth during which enzyme is most actively secreted; the production of protein is more nearly proportional to the mass of growth. During the measured active phase of hyaluronidase formation in the experiment recorded in Table 2, the ratios of enzyme potency/growth increase by factors of 3·5 and 7·4 for the two variants, whereas during this time the ratios of extracellular protein/growth increase by factors of only 1·6 and 1·7. Greater variation in the hyaluronidase ratios could, of course, be obtained if sampling were started earlier. In another experiment, for example, the hyaluronidase ratios for the same two variants increased 9·8 and 11·7 times but the protein ratios increased only 1·3 and 1·5 times. Later, when the growth rate was greatly reduced and enzyme formation had ceased, protein continued to appear. So far this latter phenomenon has not been sufficiently investigated to distinguish between cell proteins arising from autolysis of the cells and proteins truly secreted. That the protein which appears earlier in growth is unlikely to arise by cell breakdown is indicated by the absence or low concentration of nucleic acid in the supernatant. A 70 ml. sample of supernatant obtained when growth was equivalent to about 0·2 mg. bacterial N/ml. was mixed with 35 ml. of 20% (w/v) trichloroacetic acid, allowed to remain at room temperature for 60 min., and the precipitate was removed by centrifuging. This precipitate was then washed twice with 15 ml. 10% $CCl_3 \cdot COOH$ and extracted with a hot 1 : 1 mixture of ethanol and ether (20 ml.) to remove any phospholipids present. It was then dried, dissolved in 2·0 ml. of 3% NaOH, and its total phosphate content was estimated to be less than 2 μg./ml. There was 1·01 mg. protein/ml. in the precipitate as shown by the Lowry *et al.* (1951) method, and consequently the nucleic acid/protein ratio was less than 1/50. From this it seems probable that very little nucleic acid had been released into the culture fluid with the protein as would be likely if the protein arose from cell autolysis. No nucleases could be demonstrated in the culture supernatant, which might otherwise have accounted for the disappearance of liberated nucleic acid.

The amount of soluble trichloroacetic acid precipitable protein in the cultures of the less active hyaluronidase-forming variant is always slightly less than in those of the more active one. The difference, however, is not sufficient to suggest that defective protein secretion alone accounts for the variation in final enzyme potency. Unfortunately the difference between the final amounts of hyaluronidase formed by the two variants is less with casein hydrolysate media than it is with broth media.

Thus the course of formation of total extracellular proteins reflects the formation of neither coagulase nor hyaluronidase with any precision. It is much more nearly related to the growth rate than to either of the activities measured and a possible reason for this will be discussed later. It seems that the rate of formation of hyaluronidase and coagulase can be regarded as in some degree the result of specific causes.

DISCUSSION

The rate of increase of hyaluronidase in growing cultures of *Staph. aureus* first accelerates and then remains steady at a rate faster than that of growth. The growth rate itself has become constant within experimental error considerably before the early change in rate of enzyme formation is complete and remains so for a considerable time afterwards. The results are similar whether broth or acid hydrolysed casein media are used. There is no substrate or partially degraded substrate in the latter and the results are not therefore due to direct adaptive response. Moreover, the organisms form no more hyaluronidase when hyaluronate is added (Rogers, 1945). It is difficult to compare the picture with earlier studies on the rate of formation of other enzymes (cf. Gale, 1943) because many of the amino acid decarboxylases and deaminases which have been intensively studied require coenzymes and are adaptive enzymes (Gale, 1946). As Gale (1951) has pointed out, variations in enzyme formation which occur with changing pH of the culture may be related to alterations in the degree of ionization of the amino acids present in the growth medium. Also in almost all this earlier work the cultures examined had grown past the exponential phase.

It is probable that the phase during which hyaluronidase is formed faster than the organisms are growing could only last for a short time. If the period of growth at constant rate is prolonged (Novick & Szilard, 1950; Monod, 1950; Jeener, 1953; Anderson, 1953), the rate of enzyme formation and growth must eventually become equal, otherwise gross unbalance in the metabolism of the cell would result. For example, if extracellular protein were formed as rapidly as hyaluronidase, the cells would be secreting at least 13 % of their protein by the end of exponential growth instead of the observed value of only 3 %. That limited unbalance in the cell can be tolerated during growth at constant rate is in conformity with Jenner's (1953) observation that the ratio of ribonucleic acid to protein in *Polytomella caeca* cells continues to change for a time after a constant growth rate has been established. Eventually this ratio becomes constant at a characteristic value.

It is not yet known with certainty whether the early change in the cultures of staphylococci which causes hyaluronidase formation to accelerate is due to the accumulation of some essential substance or to the removal of an inhibitor. Results to be published later, however, strongly favour the first explanation, and it seems probable that this substance remains closely associated with the cells. The formal similarity between the course of production of constitutive hyaluronidase and the induced formation of enzymes may be noted.

The cessation of both hyaluronidase and coagulase formation which occurs during the period of slow growth following the exponential phase contrasts with the earlier studies (Gale, 1943) in which enzyme formation ceases only with growth. It seems unlikely that the cessation observed in the present work is a dynamic balance between formation and destruction since prolonged incubation after growth has ceased leads only to a very slow loss of hyaluronidase or coagulase.

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(*Received* 14 *August* 1953)

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Gene Interactions: the Mode of Action of the Suppressor of Acetate-requiring Mutants of *Neurospora crassa*

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SUMMARY: Certain single-gene acetate-requiring mutants of *Neurospora crassa* (*ac*) are deficient in their ability to oxidize pyruvate directly, but can decarboxylate pyruvate to acetaldehyde and can form ethanol. The growth characteristics of the *ac* mutants indicate that they can form acetate from glucose or ethanol. *ac* Mutants are inhibited by glucose or sucrose. This glucose inhibition is relieved by the single-gene suppressor mutations *sp* and *car* which lower the activity of pyruvic carboxylase, decrease the amount of ethanol formed and permit some growth of *ac sp* and *ac car* strains in absence of added acetate. The *sp* mutation also lowers ethanol dehydrogenase activity, but this activity can be partially restored by growth of *sp* strains in the presence of ethanol. As a result of the nearly complete block in their pyruvate metabolism, *ac sp* strains are forced to metabolize glucose by pathways which produce more of the initial respiratory CO_2 from the C-1 carbon of glucose.

While genetic theory is reticent about the embodiment of the gene (Pontecorvo, 1952) there is little doubt that the physiological effects of genes are mediated via enzymes. Genes, individually and *en masse*, control the rates of the step reactions from which multi-enzyme systems (Dixon, 1949) are built up, and in so doing determine the metabolic pathways chosen by the organism. In many instances gene mutation causes the block, complete or incomplete, of a step reaction; the consequence of such a block is the accumulation of precursors and the appearance of a growth requirement for the product of the blocked reaction (Beadle, 1945).

The commonest use for genetic mutants in biochemistry is the definition of routes of biosynthesis by a study of the growth requirements induced and the identification of the precursors accumulated (Horowitz, 1950). As interesting, perhaps, are the inhibitions produced by the accumulated precursors or by 'abnormal' products derived from these precursors (Emerson, 1949; Bonner, 1946). If genes do control the rates of step reactions, it should be possible with a second mutation to relieve an inhibition induced by an initial mutation, if the second mutation prevents the formation of the inhibitor. Such relief by a second mutation would be denoted, genetically, as a suppressor or modifier effect and would be one of the many instances in which the effects of two co-existing mutant genes are not merely additive. The gene interaction in such a situation would not be the simple interaction of two genes present in the nucleus but would, rather, express the interaction of the enzymic reaction systems present in non-genic parts of the cell; such interactions would further demonstrate the interdependence of the biochemical reactions of the organism. These investigations demonstrate an example of gene interaction in well-known metabolic pathways and indicate that the selection of one of several alternate pathways is here under genetic control; all this would again emphasize the importance to genetical theory of inhibition by naturally-occurring intermediates. Single-gene mutants of the Ascomycete *Neurospora crassa* which require acetate (Lein, Appleby & Lein, 1951; Lein & Lein, 1952; Strauss, 1952, 1953*a*, *b*) were used in the present work; they were selected because of the wealth of knowledge of alternate pathways of carbohydrate metabolism and because of the obvious importance of carbohydrate metabolism in the economy of *N. crassa*. These acetate-requiring mutants are inhibited by glucose. A second type of single-gene mutant with no acetate requirement was used; this latter kind of mutant is characterized by a low pyruvic carboxylase activity and lowered ethanol production as compared to the wild type. When these two mutants are genetically combined, the double mutants obtained grow slightly on media not containing acetate ('suppressor' action) and are no longer inhibited by glucose.

That the suppressor gene acts by lowering the rate of formation of an inhibitor formed as a result of the primary mutation to acetate-dependence is the conclusion drawn from these investigations.

METHODS

The technique for growing neurospora was described previously (Beadle & Tatum, 1945). Volatile or heat-labile compounds were sterilized by filtration through a Seitz filter.

Acetic acid was determined by titration of an 11 vol. steam distillate; the identity of the volatile acid was checked by Duclaux distillation when necessary. 3-Hydroxy-2-butanone [acetylmethylcarbinol (AMC)] was determined by the method of Westerfeld (1945); acetaldehyde by the method of Barker & Summerson (1941); ethanol by oxidation of volatile neutral substances with dichromate and sulphuric acid, except when it was desired to eliminate AMC in which case a preliminary distillation from alkaline mercuric salts was performed as described by Friedemann & Klass (1936). Glucose was determined by the Shaffer-Somogyi method (1933); phosphorus by the method of Fiske & SubbaRow (1925); protein N by digestion of a trichloracetic precipitate (Ballentine & Gregg, 1947); pyruvate by the method of Friedemann & Haugen (1943).

Enzyme assays were made on the supernatant from a homogenate made by suspending mycelium in three times its wet weight of cold buffer, grinding in a glass homogenizer, and then centrifuging at 0° for 10 min. at 3750 ***g***. Ethanol dehydrogenase was assayed as described by Nason, Kaplan & Colowick (1951) at pH 7·6 [tris-(hydroxymethyl)-aminomethane buffer, Sigma Chemical Co.]. Aldolase was assayed at pH 8·6 in 'tris' buffer by the method of Sibley & Lehninger (1949). This method was checked for *N. crassa* by studying the accumulation of alkali-labile phosphorus after incubation with hexose diphosphate as described below. Glucose-6-phosphate dehydrogenase was assayed by measurement of the increase in absorption of triphosphopyridinenucleotide (TPN) at 340 mμ. upon incubation with enzyme and 0·0045 M-glucose-6-phosphate substrate in pH 7·0 phosphate buffer (H. K. Mitchell, personal communication).

^{14}Carbon assays were made with a Radiation Counter Laboratories flow counter operating in the Geiger region with a gas mixture of helium + *iso*-butane in conjunction with a Tracerlab 'Utility' scaler. Self-absorption corrections were made by using an empirical curve of absorption by $CH_3{}^{14}COOH$. Counts were made at at least 10 times the background rate, and sufficient counts were recorded to insure a probable error due to the counting of less than 1 %. The 1-^{14}C-labelled glucose was obtained from the National Bureau of Standards.

MATERIALS

Organisms. The acetate-requiring mutants of *N. crassa* used in these studies were described previously under the strain designations UT 50–4, S 34, S 48, S 53, and S 210. For convenience these will be referred to as *ac-1, -2, -3, -4, -5* respectively. The low-carboxylase strain *car* (Strauss, 1952) and the suppressor strain *sp* (Lein & Lein, 1952) will be referred to by these designations. Strains *ac-2, -3, -5* have been shown to be non-allelic, while strains *ac-1* and *ac-4* can be distinguished phenotypically from the others and from each other. These latter strains will not grow on glucose + acetate as do the others but do grow on D(+)xylose + acetate. None of the *ac* mutants responds to synthetic 6,8-thioctic acid nor to yeast extract in catalytic concentrations.

Table 1. *Results of crossing* ac, sp *and* car *strains*

Genotypes of parental strains	Genotypes of segregants	Phenotypes of segregants	Total number of single ascospore cultures of each type obtained
ac-4 sp +	*ac* + *sp* *ac* + *sp* +	Grow on minimal medium	24
×	*ac-4 sp*	Grow on glucose + acetate	11
ac + *sp*	*ac-4 sp* +	Grow only on xylose + acetate	5
ac + *car* + *sp* +	*ac* + *car* + *sp* + *ac* + *car sp* *ac* + *car sp* + *ac* + *car* + *sp*	Grow on minimal medium	74
×	*ac-1 car sp* *ac-1 car* + *sp* *ac-1 car sp* +	Trace of growth on minimal medium. Grow on glucose + acetate	61
ac-1 car sp +	*ac-1 car* + *sp* +	No growth on minimal. Grow only on xylose + acetate	7

F_1 data were obtained by classifying cultures obtained from single ascospores derived from the appropriate cross. The total sample of viable ascospores is the sum of the segregants in each case.

The *sp* strain was described (Lein & Lein, 1952) as permitting some growth on minimal medium when combined with *ac-2, -3,* or *-5*. Later (Strauss, 1953*a*) it was shown that this *sp* strain carried with it low carboxylase activity with concomitant low ethanol production. It can also be demonstrated that the *sp* gene relieves the extreme glucose inhibition characteristic of *ac-4* so that *ac-4 sp* can grow on glucose + acetate (Table 1). The *sp* strain is therefore phenotypically quite similar in its action to the *car* strain, since both have low carboxylase activity and both relieve the extreme glucose inhibitions of strains *ac-1* and *ac-4*. Nevertheless, these two genes are not alleles (Table 1). The cross *ac-1 car* × *ac+ sp* leads to the formation of strains that do not grow on glucose+acetate but which will grow on xylose+acetate or on acetate alone. The simplest explanation is that *sp* and *car* are non-allelic.

Table 2. *Accumulation of pyruvic acid, ethanol and 3-hydroxy-2-butanone by resting cultures*

Cultures incubated 16 hr. in 15 ml. pH 6·0 PO_4 buffer+1 % glucose after growth for 4 days and dissimilation in buffer for 24 hr.

	Strain	
	ac-5 sp+	*ac-5 sp*
	μM/mg. dry wt. cells	
Compound accumulated		
Pyruvic acid	$1{\cdot}6 \times 10^{-2}$	$7{\cdot}4 \times 10^{-2}$
3-Hydroxy-2-butanone	$1{\cdot}9 \times 10^{-2}$	$5{\cdot}7 \times 10^{-2}$
Ethanol	7·6	2·6

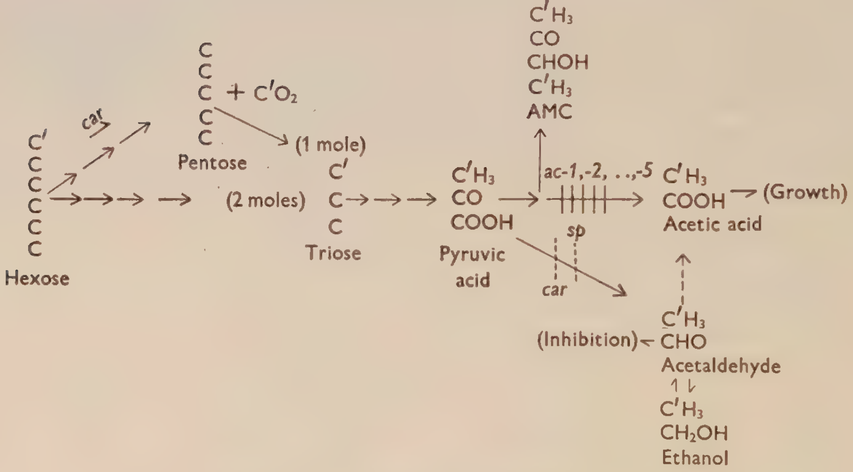

Fig. 1. Pathways of carbohydrate metabolism of importance to the *ac* mutants of *Neurospora crassa*

The strains used have the following biochemical characteristics: *ac* strains require acetate; some growth of *ac sp* or *ac car* strains does occur on minimal medium but even these strains respond to added acetate by a relatively large increase in growth rate; all *ac sp+* and *ac car+* strains are inhibited to some degree by glucose (see below); *ac* strains are deficient in their ability to oxidize pyruvic acid (Strauss, 1953*b*) and accumulate pyruvic acid and AMC (Table 2). *car* and *sp* strains have low pyruvic carboxylase activity and *ac sp* strains accumulate larger quantities of pyruvate and AMC than do *ac sp+* strains which are ethanol accumulators. These properties are summarized in Fig. 1.

RESULTS

Glucose inhibition

Strains *ac-1* and *ac-4* will not grow in the presence of glucose or sucrose but will grow on D(+)xylose+acetate. Both *sp* and *car* relieve this inhibition, and it has been suggested (Strauss, 1953*b*) that the partial block in the pyruvic carboxylase reaction, produced nutritionally by growth on xylose, or genetically by the introduction of *car* or *sp*, is biochemically involved in the relief from glucose inhibition. Strains *ac-2*, *-3* and *ac-4* grow on glucose, yet they, too, are inhibited by glucose and sucrose (Table 3). This inhibition is overcome as the culture ages and is similarly overcome by the introduction of *sp* (Fig. 2).

Table 3. *Inhibitory effect of glucose and sucrose on acetate-requiring mutants*

Medium: 20 ml. Fries+20 mg. acetate. Yield of growth recorded mg. dry wt. after 3 days growth at 25°. *Note*: *ac-1* and *ac-4* do not grow at all on sucrose or glucose but do grow with xylose+acetate.

	Strain			
Sugar	*ac-2 sp+*	*ac-3 sp+*	*ac-5 sp+*	*ac-5 sp*
	Yield of organism (mg. dry wt.)			
Glucose, 1 %	12·4	0·8	0	28·1
Sucrose, 1 %	25·7	24·2	4·0	27·0
Sucrose, 3 %	16·2	4·7	3·2	27·2
Xylose, 1 %	33·2	38·1	23·4	27·8

An attempt was made to trace the site of glucose inhibition by testing the inhibitory effects of substances related to the carboxylase reaction (Fig. 3). Pyruvate is inhibitory to *ac-3 sp+* grown in media containing 2 % sucrose but is stimulatory when tested in 1 % xylose media. Strain *ac-3 sp* is not significantly affected by pyruvate in either xylose or sucrose media. Ethanol is more inhibitory to *ac-3 sp+* on sucrose media than on xylose, and low ethanol concentrations stimulate *ac-3 sp+* slightly in xylose media. Strain *ac-3 sp* is greatly stimulated by ethanol, and the response of this strain to ethanol is identical on both xylose and sucrose media.

The response of *sp* and *sp+* strains to pyruvate and to ethanol suggests that the effect of these compounds is related and additive to the inhibitory effect of sucrose or glucose. Only the strains inhibited by sucrose are inhibited by pyruvate or by ethanol, and these compounds are not as inhibitory to *sp+* strains on xylose as on sucrose media. These considerations point to acetaldehyde (or some closely related substance) as the basis of 'glucose inhibition', since acetaldehyde is formed from pyruvate via the carboxylase reaction and from ethanol by ethanol dehydrogenase.

Acetaldehyde is inhibitory to *ac-1 car+* (Fig. 4) and to the *sp+* strains, although more acetaldehyde is required for inhibition of these strains. Because of the volatility of acetaldehyde, the concentration values given are only

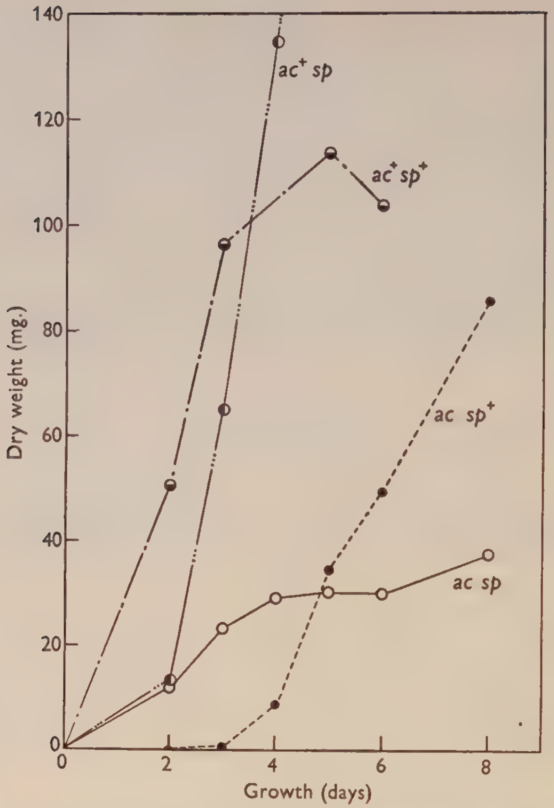

Fig. 2. Growth of *ac* mutants as a function of time. Medium: 20 ml. minimal medium (2 % sucrose) + 20 mg. acetate. *ac-3 sp* and *ac-3 sp* used; similar results are obtained with *ac-2* and *ac-5*.

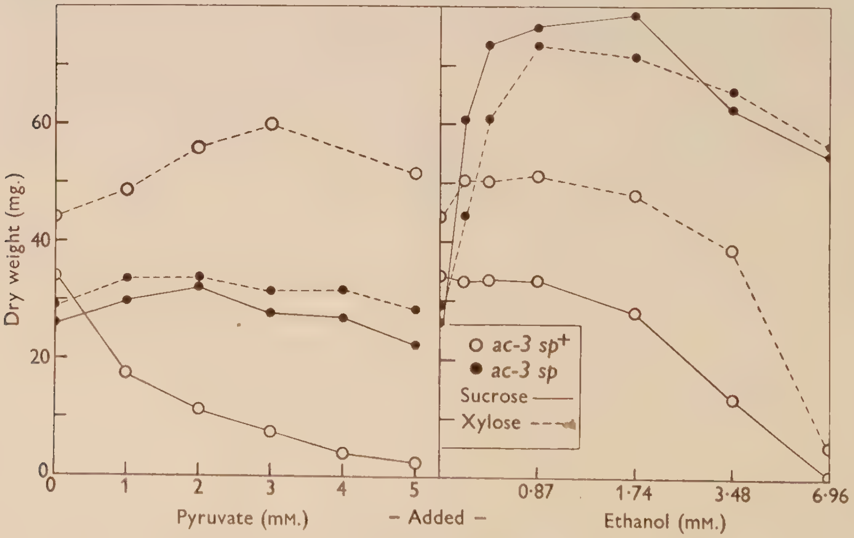

Fig. 3. Effect of added pyruvate and ethanol on *ac* strains. Medium: 20 ml. Fries + 20 mg. acetate + 1 % D(+)-xylose or 2 % sucrose as indicated. Growth period: 72 hr. at 25°.

approximate. After standing 2 days at 25° a non-inoculated medium to which acetaldehyde had been added contained almost no detectable acetaldehyde. Paraldehyde is not inhibitory to *N. crassa* at these concentrations.

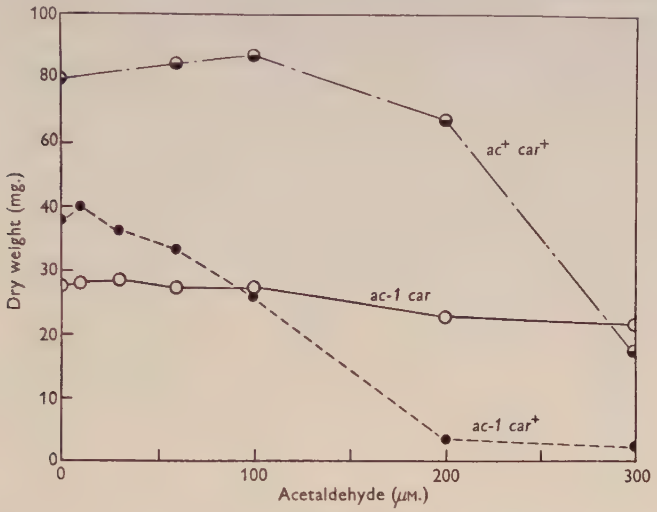

Fig. 4. Effect of added acetaldehyde on *ac* strains. Medium: 20 ml. Fries + 20 mg. acetate + 1 % xylose. Growth period: 72 hr. at 25°.

Acetate production

Lein & Lein (1952) showed that although *ac sp* strains will grow slightly on minimal medium, in a medium containing acetate *ac sp* strains grow less in a 5-day growth period than the corresponding *ac sp+* strains. This behaviour was investigated by studying the growth of *ac-3 sp* and *ac-3 sp+* strains as a function of time in media containing a limiting amount of acetate (Fig. 2). Similar growth curves are obtained with *ac-2*, *-3* and *ac-5*.

Although *ac sp* strains show growth more rapidly than *sp+* strains, growth slows after exhaustion of the added acetate from the medium at 3–4 days. The lag which *ac sp+* strains show before growth is initiated is probably due to inhibition by sugar in the medium. Once this inhibition is overcome, *ac sp+* grows at a rate approaching that of the wild type. No appreciable volatile acid accumulates in the medium during the rapid period of growth after the *ac sp+* strain has overtaken the *ac sp* strain. The rapidly growing *ac sp+* is still an acetate-requiring mutant since subcultures of the rapidly growing mycelium made on acetate medium and then transferred to minimal medium do not grow unless acetate is added. Decreasing the amount of acetate added to *ac sp+* strains has no effect on the total crop of mycelium eventually obtained. Cultures of *ac-3 sp+* fed one-fortieth the amount of acetate which produces maximum growth in a 4-day period eventually attained the wild-type yield of mycelium, although over 2 weeks were required. The mycelium obtained in this last case was shown to be that of an acetate-requiring mutant.

ac sp+ Strains accumulate considerable amounts of ethanol as compared to their *ac sp* counterparts, and the rapid increase in dry weight of *sp*+ strains is accompanied by the appearance of ethanol in the medium (Fig. 5). The addition of ethanol to *ac sp* cultures after 2 days growth causes them to duplicate the rapid growth characteristic of *ac sp*+. In the absence of other carbon sources all *ac* strains give a few milligrams of growth with ethanol as well as with acetate. In the presence of sugar, ethanol can be used as a substitute for acetate by *ac sp* strains (Fig. 6), but no growth of *ac sp*+ on ethanol in the absence of acetate has ever been obtained. A previous report (Strauss, 1952) that ethanol could not be used for growth initiation by *ac-1 car* was due to the use of a standard 3-day growth period. There is a lag before growth begins on ethanol, and this seems to be related to the time required for the appearance of a visible amount of growth from a control inoculum on minimal medium. It is also evident from the data that the effect of ethanol and acetate together is more than additive.

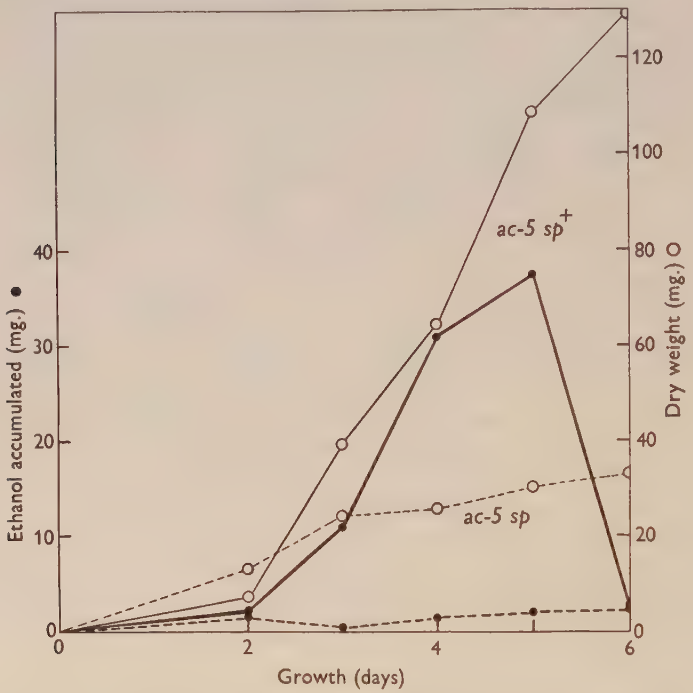

Fig. 5. Ethanol accumulation and growth as a function of time for *ac* strains. Medium: 20 ml. minimal (2% sucrose)+20 mg. acetate.

These data indicate that acetate (or its active form) can be synthesized from glucose or from ethanol by *ac* mutants. Further evidence for this hypothesis is given in Table 4. Glucose lowers the amount of acetate taken up by *ac sp*+ strains but not by *ac sp* strains, which require the addition of ethanol in order to decrease acetate uptake. This is not a case of glucose inhibition, since it can be shown that *ac sp*+ strains take up a greater amount of glucose than do *ac sp*.

An attempt was made to demonstrate the formation of acetate by utilizing the methods of isotope dilution. No significant dilution of added $CH_3{}^{14}COOH$ could be demonstrated when cultures of *ac sp+* were grown for 4 days in labelled acetate + glucose, nor could any acetate of constant specific activity be isolated from cultures fed acetate + glucose-1-^{14}C. Wild-type cultures also failed to give evidence for acetate synthesis under these conditions. These experiments are therefore not considered evidence against the hypothesis of acetate formation by acetate-requiring mutants.

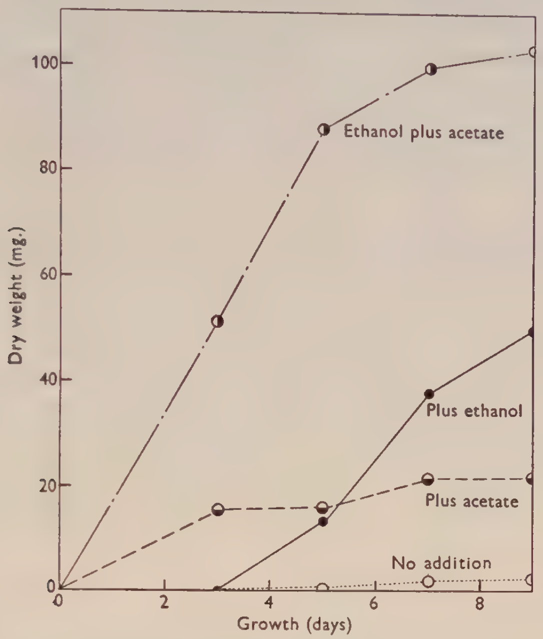

Fig. 6. Effect of ethanol on growth of *ac-1 car*. Medium: 20 ml. minimal (2 % sucrose). Ethanol, 40 mg. added. Acetate, 10 mg. added.

Table 4. *Uptake of acetate by ac mutants*

	Strain	
	ac-3 sp+	*ac-3 sp*
	mg. acetate taken up/mg. av. dry wt.*	
Suspending medium		
Buffer + 100 mg. acetate	0·20	0·23
Buffer + 100 mg. acetate + 2 g. glucose	0·13	0·24
Buffer + 100 mg. acetate + 2 g. glucose + 250 mg. ethanol	0·15	0·19

* Average dry wt. = wt. at 0 time + wt. after 22 hr. incubation/2.

Alterations in metabolic pattern

Previous work (Strauss, 1953*b*) indicated that the lowering of pyruvic carboxylase activity by either genetic means (introduction of *car*) or nutritional means (growth on xylose) relieved the inhibition of *ac* strains. *car*+ Strains have 4 to 6 times the carboxylase activity of *car* strains after growth on sucrose, but have at most twice the activity of *car* strains after growth on xylose. *car* Strains have the same carboxylase activity after growth on either sucrose or xylose. It was therefore suspected that the *car* gene (and analogously the *sp* gene) might act by shunting metabolism to pentose. This would lead to the same effects on enzyme pattern as growth on xylose: lowered carboxylase formation with consequent relief from glucose inhibition. This idea was tested using glucose 1-^{14}C. If *sp* and *car* strains metabolize glucose via a pentose shunt, then one would expect a relatively higher respiratory CO_2 and a lower molar radioactivity in the ethanol accumulated by *car* strains than in that formed by *car*+ strains, since in the operation of the Embden-Meyerhof scheme the C_1 of glucose is retained in the methyl of pyruvate, AMC and ethanol (Fig. 1). The exact values obtained would be complicated by the factors of assimilation and CO_2 fixation. These probably occur in the 18–20 hr. incubation period under aerobic conditions which is necessary to accumulate sufficient quantities of ethanol. For the comparison of different strains the ratio of ethanol activity to respiratory CO_2 activity has been used as the most obvious method.

Mycelial pads were obtained after 4 days growth at 25° in 20 ml. minimal medium containing 20 mg. acetic acid. The pads were then transferred to 20 ml. pH 6·0 M/15 phosphate buffer and allowed to dissimilate for 24 hr. Two or three pads were then transferred to 125 ml. flasks containing 20 ml. 1·5 % glucose $-^{14}C_1$ in M/30 phosphate buffer. An inverted U tube containing soda lime was attached to the flasks and a cup containing 4 ml. 2N-sodium hydroxide was suspended above the mycelium. The cultures were not shaken and it was not supposed that this method diminished the CO_2 tension of the medium to zero. After 18–20 hr. incubation the absorbed CO_2 was precipitated as $BaCO_3$. The medium was neutralized, steam distilled, and the steam distillate was redistilled from a mixture of alkaline mercuric salts. A sample of this distillate was oxidized with acid dichromate and steam distilled. The distillate was neutralized with NaOH, plated, and counted as sodium acetate. The specific activity of the glucose used was determined from its osazone. In all cases some glucose remained in the medium at the conclusion of the experiment.

ac car and *ac sp* Strains gave higher respiratory CO_2 and lower ethanol activities than did their *ac*+ counterparts (Table 5). This does not seem to be an effect of the *sp* gene acting alone since *ac*+ *sp* gives activities closer to the wild-type and to *ac sp*+ values than to *ac sp*. There can be little doubt that *ac sp* strains metabolize glucose over quantitatively different pathways from those used by the wild-type, but this seems due to neither *ac* nor *sp* gene alone but rather to their combination. The *car* gene behaves more nearly as predicted, since *ac*+ *car* gives a much lower ratio than does the wild-type and approaches the ratio given by *ac car*. In all cases studied the double mutants *ac sp* or *ac car* give lower ratios than does any single mutants.

If the Embden-Meyerhof pathway were solely responsible for ethanol formation by any of the strains tested, the isolated ethanol should have an activity of 25×10^4 counts/min./mM. Dilution of this by ethanol formed via carbon dioxide fixation or via the pentose shunt would lower the value obtained. It is possible that the low values obtained are the result of such dilutions. The exact values obtained may depend on the particular genetic background of the strain employed, since it is possible that all the factors leading to dilution of the labelled ethanol are genetically controlled.

Table 5. *Genetic alteration in the pattern of glucose metabolism*

Recorded counts/min./mM $\times 10^{-4}$. Glucose activity corrected to 5×10^5 counts/min./mM. Values are averages of duplicate experiments run simultaneously. *ac-1 car+* and *ac-1 car* grown on 1% xylose + acetate.

	Strain						
	ac+ car+ sp+	*ac+ sp*	*ac-3 sp+*	*ac-3 sp*	*ac+ car*	*ac-1 car+*	*ac-1 car*
	counts/min./mM $\times 10^{-4}$						
Respiratory CO_2	2·0	2·5	2·2	3·7	2·5	1·9	3·0
Ethanol	12·7	14·6	18·0	9·7	8·9	12·7	9·7
Ratio: Ethanol/CO_2	6·4	5·8	8·2	2·6	3·6	6·7	3·2

Enzymic pattern

To verify the results obtained in growth and isotope experiments, enzymic assays for ethanol dehydrogenase, aldolase and glucose-6-phosphate dehydrogenase were undertaken. Aldolase was studied because it is a key enzyme in the Embden-Meyerhof scheme, glucose-6-phosphate dehydrogenase because it is the first step in the pentose shunt, and ethanol dehydrogenase because of its obvious relation to accumulation and utilization.

Enzyme activity was measured after growth for 4 days on sucrose or xylose (Table 6). Ethanol-dehydrogenase assays were also made using cultures of *ac-3 sp* and *ac-1 car* grown 3 days in the presence and absence of added ethanol (Table 7). It will be noted that the expected correlation between ethanol accumulation and ethanol dehydrogenase activity was obtained. Strains *ac sp* and *ac-1 car*, which accumulate very little ethanol, have low ethanol dehydrogenase activities. When grown in the presence of ethanol, however, the dehydrogenase activity of these strains is increased, although it still does not approach the wild-type level (Table 7). This is in contrast to the effect of pyruvate on these strains. Both have low pyruvic carboxylase activities but growth with added pyruvate does not increase their carboxylase activities. All the mutant strains tested have lower ethanol dehydrogenase activities than the wild-type, in accordance with the findings of Nason, Kaplan & Oldewurtal (1953).

Aldolase activity is given as μM of hexosediphosphate (HDP) split/hr./mg.

protein N. HDP values were obtained as described by Sibley & Lehninger (1949) by correlating the triose chromogen readings of their method with the alkali-labile P liberated. *N. crassa* does contain aldolase, and a linear correlation is obtained between enzyme concentration, alkali-labile P production and chromogen reading. The aldolase activities of the strains used in these investigations are similar with the exception of *ac-4 sp+* which has low activity (Table 6) and there is no difference in activity after growth on xylose as compared to growth on sucrose. The lower wild-type aldolase activity may be due to the disappearance of sugar from the medium in which this strain is growing, after 3 days of growth.

Table 6. *Enzyme activities of ac strains*

Aldolase activity: μM-HDP split/hr./mg. protein N at 37°. Glucose-6-phosphate dehydrogenase activity: increase in optical density at 340 mμ./min./mg. protein N. Ethanol dehydrogenase activity: increase in optical density at 340 mμ./min./mg. protein N.

Strain	Aldolase	Enzyme, glucose-6-phosphate dehydrogenase	Ethanol dehydrogenase
1. Grown 4 days on 2 % sucrose			
ac+ car+ sp+	0·44	0·52	1·43
ac+ car	0·55	0·22	0·069
ac-1 car	0·80	0·20	0·007
ac+ sp	0·65	0·017	0·043
ac-2 sp+	0·65	0·11	0·14
ac-3 sp+	0·80	0·21	0·12
ac-3 sp	0·75	0·13	0·004
ac-5 sp+	0·75	0·16	0·24
ac-5 sp	0·80	0·15	0·0
2. Grown 4 days on 1 % xylose			
ac+ car+ sp+	0·55	0·22	0·20
ac-1 car+	1·0	0·044	0·01
ac-1 car	0·65	0·12	0·009
ac+ sp	0·55	0·0	0·001
ac-4 sp+	0·13	0·058	0·047

Table 7. *Effect of ethanol on ethanol dehydrogenase activity*

Recorded activity/mg. protein N × 10^3 after 3 days growth at 25° in 20 ml. minimal medium + acetate + addition as given.

Strain	Addition to medium: None	Addition to medium: Ethanol (190 mg.)
ac+ sp+	570	490
ac-3 sp	0	58
ac-5 sp	4	66

The *ac sp+* and *ac car+* strains used have at most 1/2 to 1/3 of the glucose-6-phosphate dehydrogenase activity of wild-type. There is no difference in the activity of *ac sp* and *ac sp+* strains. The *ac+ sp* strain used in these investigations has an extremely low glucose-6-phosphate dehydrogenase activity, in marked contrast to the *ac+ car* strain which has an activity similar to the other mutants. Enzyme activity is lower after growth on xylose than after growth on sucrose, but the *ac-1 car* strain has three times the activity of *ac-1 car*+ after growth on xylose. Since no genetic studies were made, these strain differences are not necessarily characteristic of the *sp* and *car* genes.

DISCUSSION

The following hypothesis accounts for the data (Fig. 1). *ac* Mutants cannot oxidatively decarboxylate pyruvate to acetate (or active acetate). AMC accumulates. The pathway pyruvate → acetaldehyde → acetate is open and *ac* mutants make acetate via this route. In the early stages of growth *ac* strains do not remove acetaldehyde rapidly enough. Acetaldehyde, or some substance formed from acetaldehyde, is inhibitory and is the cause of 'glucose inhibition'. After some time certain of the *ac* strains acquire the ability to remove the formed acetaldehyde at a rate sufficient to keep its concentration below the inhibitory level. At that time, rapid growth ensues and continues, presumably on the acetate formed from acetaldehyde oxidations.

The introduction of either the *sp* or *car* gene into *ac* strains results in relief from glucose inhibition, since a decrease of carboxylase activity induces a greatly lessened rate of acetaldehyde formation. Some acetaldehyde is still formed, however, and the oxidation of this to acetate leads to slow growth and the partial 'suppression' of the *ac* mutants.

Lowering the rate of acetaldehyde formation lowers the ethanol dehydrogenase activity of *car* and *sp* strains by eliminating the substrate upon which this dehydrogenase acts. This is an example of the mechanism by which a single gene change can lead to a change in the activity of more than one enzyme by indirect means, since growth of *ac sp* strains in the presence of ethanol partially restores enzyme activity (cf. Vogel & Davis, 1952).

Prohibition of ethanol formation by *ac*+ strains increases the crop of mycelium obtained; *ac*+ *sp* strains have given nearly twice the dry weight of *ac*+ *sp*+ strains under our conditions. There is a slight retardation of the initial growth rate caused by the *sp* gene. Apparently the 'ethanol excursion' (Foster, 1949) is not a particularly efficient mechanism, but *ac*+ *sp*+ strains have no method for controlling the amount of glucose they take up and must consequently accumulate ethanol.

This hypothesis is consistent with the growth data; the greater susceptibility of *ac sp*+ strains to ethanol and pyruvate inhibition is explained by the ability of these substances to serve as acetaldehyde precursors. The *ac* strains may be more susceptible to acetaldehyde poisoning, rather than being poisoned by amounts of acetaldehyde greater than normal. Decision rests upon location of the precise biochemical lesions in pyruvate oxidation in the different non-allelic acetate mutants. While some compound derived from acetaldehyde may be the actual inhibitor, because of the relatively high concentrations required for inhibition, the relationship to acetaldehyde must be close, since the non-allelic genes *sp* and *car* both relieve glucose inhibition and are both associated with low carboxylase activity and since no inhibition is noticed when growth occurs on xylose, which similarly leads to low carboxylase activity.

Altered pathways of metabolism. Although *ac sp* mutants do form more of their initial respiratory CO_2 from the 1-carbon of glucose than do *ac sp*+ strains, there is no evidence that this is a direct result of the *sp* mutation. Apparently the introduction of a double block in the pyruvic acid metabolism slows down glucose metabolism and retards the steady-state operation of the Embden-Meyerhof path. Since glucose can no longer be metabolized easily by this route, an alternate pathway is used to a relatively greater extent, giving the 1-carbon of glucose as CO_2. If this alternate path utilizes the action of glucose-6-phosphate dehydrogenase, then *ac sp* strains are not able to increase the amount of enzyme sufficiently to take care of glucose at the total wild-type rate. Pyruvate is accumulated by *ac sp* strains to a greater extent than by *ac sp*+, but if the above interpretation is correct, the pyruvate accumulated by *ac sp* has a different metabolic history than the lesser amounts of pyruvate accumulated by the *ac sp*+ strain. The *car* gene, however, seems to act by shunting metabolism along an alternate route in accordance with the original hypothesis, since the *ac*+ *car* strain tested has a lower ethanol/CO_2 activity ratio than the wild-type. If these interpretations are correct, then *car* and *sp*, which are genetically non-allelic, lower carboxylase activity by different mechanisms.

Mechanism of suppressor action. The mechanism for suppressor action derived from these studies is very similar to that proposed by Emerson (1948) for suppression of the *sfo* characteristic. It was supposed by Emerson and later by Horowitz (1951) that this mechanism was not of general applicability. However, it would seem that this type of suppressor action might well be more common than is supposed, especially since so many single gene mutations seem to result in inhibition by normal metabolites. Suppressors may often be 'loss' mutations which prevent the formation or accumulation of inhibitory substances formed as a result of a primary mutation. Since such suppressed mutants have essentially two biochemical lesions it might be expected that they would approach but not reach the true wild-type phenotype as is the case for the *ac sp* mutants. Giles (1951) and Giles & Partridge (1953) described certain suppressors of the methionineless and inositolless characteristics that similarly approach but do not reach the wild-type phenotype. Similarly, Mitchell & Mitchell (1952) described a whole series of inhibitory phenomena connected with the action of a suppressor gene.

The authors are indebted to Miss M. L. Jezyk and to J. E. Matheson for technical assistance, and to Dr S. H. Hutner for helpful criticism of the manuscript.

The work was supported by contract AT (30–1)–1138 between Syracuse University and the (U.S.) Atomic Energy Commission.

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(*Received* 17 *August* 1953)

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The Association of the Penicillin-binding Component of *Staphylococcus aureus* with a Lipid Fraction

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SUMMARY: Radiopenicillin is strongly bound by ultra-microscopic lipid-containing particles liberated on mechanical rupture of *Staphylococcus aureus* cells. The binding resembles that of intact cells in that it is irreversible and only occurs to a limited extent, but differs in that 7–12 times as much penicillin is bound per unit dry weight of material. The supernatant after centrifuging down the lipid particles decreases the titre of added penicillin as indicated by diffusion assay, possibly by a small irreversible inactivation superimposed upon a 'reversible' type of binding.

There is a correlation between the distribution of (*a*) the penicillin-binding component (P.B.C.), (*b*) ^{35}S from radiopenicillin pretreated cells, and (*c*) lipid phosphorus, in the three fractions produced on rupture either in distilled water or in a formaldehyde solution. Rupture in formalin appears to allow the cells walls to retain most of the lipid particles and P.B.C., but only a little extra of the dry weight of the cells. Thus penicillin reacts with a lipid-containing fraction close to the cell wall in intact organisms. At least as much more P.B.C. is liberated on rupture of the cells as was available to the penicillin in the intact cell, but P.B.C. is somewhat unstable after rupture. These data are discussed in the light of evidence in the literature that penicillin may react initially with the osmotic barrier of bacteria.

It was noted several years ago in this laboratory that staphylococcal cell walls prepared by mechanical rupture of cells in distilled water (Cooper, Rowley & Dawson, 1949) were quite white, and that most of the yellow pigment present in the supernatant fraction was sedimented by high-speed centrifugation as a thin orange waxy layer coating the surface of the cell-wall layer. The lipid character of this fraction was suspected from its yellow colour, as nearly all the pigments of staphylococci when extracted by 90% phenol in water were soluble in chloroform. These pigments have been reported to be carotenoid in character (Sobin & Stahly, 1942). Mitchell & Moyle (1951*b*) confirmed the high proportion of phospholipid in the coloured fraction which sediments more slowly than the cell walls when a mechanically ruptured cell suspension is centrifuged.

It has been reported elsewhere (Rowley, Cooper, Roberts & Lester Smith, 1950) that penicillin-sensitive cells contain a penicillin-binding component (P.B.C.) which appears to be related to the mode of action of penicillin. Cell walls prepared in a formalin mixture were able to bind penicillin (Few, Cooper & Rowley, 1952), whereas cell walls prepared in distilled water were not (Cooper *et al.* 1949). It was noticed that cell walls which could bind penicillin were yellow and those which could not were quite white and that this colour difference always followed the penicillin-binding capacity. It was, therefore, considered possible that P.B.C. was associated with a lipid fraction of the cells, and the results presented below show that this is the case.

METHODS

Measurement of penicillin uptake by intact cells and cell walls using radiopenicillin has been previously described (Rowley *et al.* 1950; Few *et al.* 1952). Uptake by 'lipid particles' (see below) was measured as described in Table 1. The purity of the radiopenicillin was checked by the methods described by Cooper, Clowes & Rowley (1954).

Cell preparation. A penicillin-sensitive *Staphylococcus aureus* was grown on tryptic digest agar in Roux bottles for 16–40 hr., harvested in neutral 0·05 M-phosphate solution, filtered through a no. 1 porosity sintered glass funnel to remove agar particles and washed 3 times with distilled water. The organisms were used at once or were freeze dried.

Disruption of cells. Cells were disrupted in distilled water or in 'medium A', which consisted of 1 ml. formalin solution, 0·1 ml. mercaptoacetic acid, 0·5 g. citric acid and 50 ml. distilled water saturated with octyl alcohol, made up to 100 ml. with water and adjusted to pH 6·5. Ten ml. of a cell suspension were agitated with 4 g. of glass ballotini beads, no. 12, with a Mickle shaker (Mickle, 1948; Mitchell & Moyle, 1951*b*). The concentration of cells was up to 25 mg. dry wt./ml. for distilled water, or up to 15 mg./ml. for medium A since the latter did not allow complete formation of typical penicillin-binding cell walls at concentrations higher than this. The rate of rupture was followed by a turbidimetric method (Cooper, 1953). After shaking for 1 hr. 10–15 % of the organisms remained intact with distilled water as shaking mixture and 20 % with medium A. Little further rupture occurred on continued shaking. In all quantitative analytical work unruptured cells present in the cell-wall preparation were allowed for.

Separation of fractions after cell rupture. The suspension of ruptured cells was removed by a pipette, and when quantitative recovery was required the ballotini were washed 5 times with 2 ml. portions of distilled water. The suspension was spun for 15 min. at 2500 ***g*** to remove cell walls and intact cells, and the supernatant was re-centrifuged twice at this speed to sediment cell wall debris which was added to the cell wall fraction. The supernatant, still quite turbid, was centrifuged for 30 min. at 20,000 ***g*** to sediment the 'lipid particle' fraction, which appeared as a homogeneous orange translucent pellet free from opaque white cell wall debris. No lipid particles were freed in medium A at concentrations below 15 mg. dry wt. bacteria/ml., but the yellow cell walls formed in this case had to be separated from a white heavier layer which appeared to be denatured protein and which contained no P.B.C., or ^{35}S from radiopenicillin pretreated cells. The separation was easily effected with a Pasteur pipette followed by differential centrifuging. The white layer was added to the supernatant fraction for assay purposes.

Biological penicillin assays used an agar plate diffusion method with *Sarcina lutea* as test organism, porcelain cylinders of 8 mm. diameter, and an incubation time of 16 hr. at 27° (Welch, 1948). Penicillin solutions containing 0·02 and 0·05 u./ml. gave inhibition zones of 20 and 30 mm. diameter respectively so that the decrease in titre of about 0·01 u./ml. observed in presence of the supernatant fraction was well outside experimental error. Tenfold replicates were used.

Assay of lipid phosphorus. The procedure followed was similar to that described by Mitchell & Moyle (1951*b*). The weighed freeze-dried samples (*c.* 20 mg.) were refluxed for 1 hr. with 2 ml. methanol in 20 ml. tubes with B14 sockets and condensers and the cooled extracts were transferred to fresh tubes. This procedure was repeated twice and the pooled methanolic extracts were evaporated to dryness and kept overnight *in vacuo* over P_2O_5. The dried extracts were then re-extracted overnight at room temperature with 3 ml. Na-dried ether, and the ether extracts transferred to fresh tubes; this step was also repeated twice. The pooled extracts were evaporated to dryness and the fatty residues digested with 0·5 ml. conc. HNO_3 (AR) + 1 drop of 100 vol. H_2O_2 until colourless. The total phosphate in these solutions was estimated by the colorimetric method of Fiske & SubbaRow (1925).

RESULTS

Penicillin binding by lipid particles

The lipid particle fraction obtained by high-speed centrifuging of the cell wall-free 'cytoplasmic contents' of the cells after rupture in distilled water was found to bind penicillin strongly (Table 1), the uptake being 10–15 u./g., i.e. 7–12 times greater than the original cells. It can be seen that this uptake is independent of penicillin concentration and is completely prevented by pre-treatment of the cells with a small amount of non-radioactive penicillin. The uptake by the lipid particles is thus similar to that by intact cells in that it appears to be an irreversible reaction with a component present in limited amount.

Table 1. *Uptake of radiopenicillin on lipid particles before and after addition of* 0·2 *u. ordinary penicillin/ml.*

Radiopenicillin was added to the unwashed suspension of lipid particles in the 'cytoplasmic fluid', and after 20 min. at room temperature the particles were centrifuged for 30 min. at 20,000 *g* and resuspended in distilled water. The uptake was determined as the amount of ^{35}S removed from this once-washed suspension by a second centrifugation. The second deposit was weighed.

Conc. radio-penicillin before washing (u./ml.)	Untreated			Pre-treated with ordinary penicillin		
	^{35}S in once-washed suspension (counts/ml./min.)	^{35}S in supernatant after second centrifuging (counts/ml./min.)	Radio-penicillin uptake (u./g.)	^{35}S in once-washed suspension (counts/ml./min.)	^{35}S in supernatant after second centrifuging (counts/ml./min.)	Radio-penicillin uptake (u./g.)
0·1	1410	390	9·0	100	130	$<0·3$
0·2	1650	420	10·6	190	220	$<0·3$
0·3	1730	610	9·7	320	320	$<0·3$
0·4	1960	800	10·0	350	340	$<0·3$

The significantly high value of the supernatant radioactivity of the untreated preparation (col. 3, Table 1), compared with that of the penicillin pre-treated controls (col. 6), suggests that about 20% of the P.B.C.-radiopenicillin complex present in the lipid particles may be soluble, and indeed it has been found that less material is precipitated after the second centrifugation than after the first; thus 5–6% by weight of the bacteria appeared as lipid particles (Table 2), but only 3–4% was left after one wash. Resuspension in a larger volume of water (50 ml.) was sufficient to dissolve the lipid particles completely, leaving no deposit on centrifuging.

The decrease in penicillin titre caused by lipid particles when resuspended in distilled water, as determined by biological assay, agreed well with the uptake of radiopenicillin. When the concentration of lipid particles was about 1 mg. dry wt./ml., about 25% of this biologically assayed P.B.C. was still present in the supernatant after centrifuging for 1 hr. at 20,000 ***g***. A similar proportion of P.B.C. (about 30% of the total recovered) was present in the supernatant fraction after rupture (see below). These data indicate that the amount of P.B.C. present in the lipid particles which will dissolve in 1 ml. will bind about 0·01 units of penicillin.

Decrease in penicillin titre caused by the supernatant fraction

In order to measure the amount of P.B.C. in the clear supernatant from high-speed centrifugation after rupture in distilled water, standard penicillin solutions were equally diluted in the supernatant and in distilled water, placed at once on the assay plates and incubated. It was found that this supernatant fraction decreased the titre of the penicillin solutions to an extent which was dependent on penicillin concentration (Fig. 1). The straight-line portion could be extrapolated to cut the vertical axis at 0·75 u. penicillin/g. dry wt. of cytoplasmic contents (*c.* 0·01 u. penicillin/ml. supernatant). At low penicillin concentrations only a small proportion of the penicillin which had been added was still detectable, whereas this proportion was much larger at higher penicillin concentrations. Fig. 1 suggested that the irreversible and limited binding by the small amounts of soluble P.B.C. which were expected to be present was superimposed on a different type of penicillin inactivation. To see whether this was enzymic, in which case further incubation should decrease the titre even more, the supernatant fraction was incubated with penicillin for 6 hr. before adding to the assay cups. As the very small further decrease in titre then observed was only of the same order as that of a control in distilled water, it appeared more likely to be due to binding, perhaps by protein, for example. If such were the case, the binding was reversible, as estimation of bound radiopenicillin by removing excess radiopenicillin from the supernatants by an electro-dialysis method (Few, Cooper & Rowley, 1953, and unpublished results) gave a straight line of very much lower slope. As this line passed through the origin no P.B.C. was detectable, and the P.B.C. must therefore be considered to be dialysable. The rate of dialysis of the radiopenicillin was also much slower in the presence of supernatant material than in its absence, suggesting that the removal of the penicillin ions was being hindered by a reversible binding.

It is interesting that the reversible binding by supernatant material is very large compared with the irreversible binding by intact cells (Table 2). No reversible binding of a similar order by the intact cell was found in this present work or by Maass & Johnson (1949*a*, *b*) or by Rowley *et al.* (1950), using

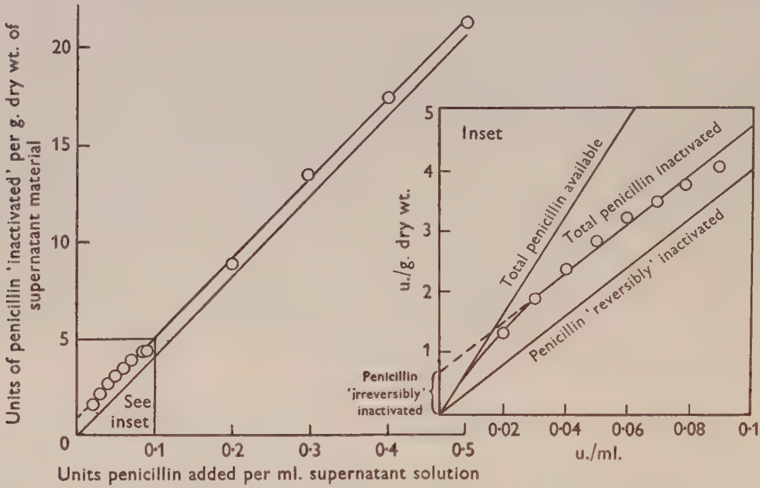

Fig. 1. Decrease in penicillin titre caused by the supernatant fraction obtained by centrifuging ruptured suspensions of *Staph. aureus* (20 mg. dry wt./ml.) at 20,000 *g* for 30 min. The suspension was removed from the ballotini beads without dilution. 'Total penicillin available' represents the amount of penicillin inactivated if the penicillin titre was decreased to zero.

Table 2. *Distribution of lipid* P, P.B.C. *and* ^{35}S (*from radiopenicillin pre-treated staphylococci*) *in the three fractions obtained on mechanical rupture*

P.B.C. and ^{35}S are expressed as equivalent units of penicillin/g. dry wt. of fraction, lipid P as μmole PO_4'''/g. Two values in a space indicate the range observed in several experiments. All percentages are in terms of intact cells.

	% dry wt	P.B.C.		^{35}S		Lipid PO_4'''	
		%	u./g.	%	u./g.	%	μmole/g.
		Ruptured in distilled water					
Intact cells	(100)	(100)	1·2–1·7	(100)	1·2–1·7	(100)	32·2
Cell walls	20–28	0	0	0–2	<0·01	<2	<0·5
'Lipid particle' fraction	5–6	60–75	10–13·6	19·5	5·3	17·2	100·5
Supernatants	60–66	Probably 30	Probably 0·75	69	1·6	70	35
Totals	85–90	90–105	—	90	—	88	—
		Ruptured in 'medium A'					
Intact cells	(100)	(100)	2·2	(100)	1·5	(100)	32·2
Cell walls	33–40	60–75	4·5–6	75	2·9	63	77
'Lipid particle' fraction	1–2	*	*	<3	<3	8	128
Supernatants	60–70	*	*	12·5	0·3	16	9·3
Totals	95	60–75 at least	—	88	—	87	—

* No suitable assay

biological and radioactive assay methods. Thus the reversible binding centres appear to be liberated on rupture. Reversible binding of penicillin G (Klotz, Urquhart & Weber, 1950) by bovine serum albumin, when expressed as u. penicillin/g. dry wt., is of a similar order to that of the supernatant fraction in the present case.

Correlation between distribution of penicillin-binding component from untreated cells, ^{35}S from radiopenicillin pre-treated cells, and lipid phosphorus

Table 2 shows that P.B.C., ^{35}S and lipid P are practically absent from cell walls prepared in distilled water, present in the lipid particles to a high concentration and in the supernatant fraction to less than or the same amount as in intact cells. The proportions of ^{35}S closely followed the proportions of lipid P, and the proportions of P.B.C. are similar, although greater differences may be expected here since some extra P.B.C. is produced on rupture (see below) and the moderate stability of P.B.C. when in solution may differ from that of the lipid particles. A similar correlation was found in medium A, where the bulk of the P.B.C., ^{35}S and lipid P were retained in the cell wall while only a small extra proportion of the dry weight was retained.

Differences in rupture products obtained from distilled water and medium A

In medium A the 'lipid particle fraction' was 1–2 % as compared with 5–6 % in distilled water (Table 2). In fact, the small sediment observed was not at all similar to the lipid particles from distilled water, being neither orange nor waxy, but white and opaque and resembling finely divided cell-wall material. The supernatants from medium A were practically colourless, whereas those from distilled water were yellow. On the other hand, the cell walls from medium A were orange, whereas those from distilled water were white.

Effect of lipid solvents

Refluxing for 1 hr. with methanol or pyridine, or standing in 90 % (w/v) phenol in water removed the bulk of the ^{35}S from radiopenicillin pre-treated cells. Refluxing dried organisms 3 times with 3 ml. MeOH for 5 min. (under N_2) destroyed or extracted all the P.B.C. from the cells, together with a considerable amount of lipid material. Unfortunately, whether destruction or extraction had occurred could not be decided as the extraction products interfered with the biological assay.

High recovery of penicillin-binding component after rupture in distilled water

Table 2 shows that, while the recoveries of material measured by dry weight, ^{35}S and lipid P were 85–90 %, the recovery of P.B.C. was always at least 90 %. This was surprising since cell-free P.B.C. was found to be rather unstable, and 2–2½ hr. had elapsed between the start of rupture and the time of assay. Also, the uptake by lipid particles represented only that amount of penicillin remaining bound after one wash in distilled water, and as shown above P.B.C. is slightly soluble. This suggested that more P.B.C. was liberated on rupture than was available to the penicillin in the intact cells, an idea which was supported by the finding that the penicillin ^{35}S bound by lipid particles was 2–3 times as much per unit dry weight as the amount of ^{35}S recovered in the lipid particle fraction from cells saturated with radiopenicillin before rupture (Table 2, cols. 3 and 5). When 0·3 u. radiopenicillin/ml. was present during cell rupture in distilled water instead of being added afterwards, twice as much radiopenicillin was bound by the lipid particles (i.e. 19–27 u./g. compared with 10–13 u./g.). Presumably the penicillin reacted with the P.B.C. immediately it was formed, thus avoiding any inactivation of the P.B.C.

The high recovery of P.B.C. was confirmed by pre-treating the cells with non-radioactive penicillin and removing the excess by thorough washing. The uptake of radiopenicillin by a sample of the intact cells was then less than 0·08 u./g., yet on rupturing the remainder the uptake by the lipid particles was 4–5 u./g., i.e. about the same as when radiopenicillin was added, and the excess removed, before rupture. The recovery of P.B.C. in the lipid particle fraction alone in this particular experiment, by radioactive assay, was then greater than 400 % of that available to the penicillin in the starting material. These results indicate that considerably more P.B.C. is liberated on rupture of the cells than could react with the penicillin before rupture, and that P.B.C. is somewhat unstable during rupture.

DISCUSSION

The results presented above show that the component in staphylococci which binds penicillin is associated with a lipid fraction situated close to the cell wall of intact organisms, and suggest that this penicillin-binding component may itself be a lipid. However, this may not be the case, as, calculated on the basis of the lipid phosphorus, the lipid particles would only contain about 10 % of material of the composition of lecithin. The lipid particles ('small particle fraction') prepared from a *Staphylococcus aureus* strain by Mitchell & Moyle (1951*b*) contained components other than lipids, some of which, as these authors pointed out, were very similar in composition to the cell-wall material which did not bind penicillin. They suggested that the small particle fraction was the lipid layer observed cytologically under the cell wall. Their analyses for this fraction were, in fact, consistent with 20–30 % fat-soluble material, 30–70 % of adhering cell-wall material and a few per cent of substances (e.g. nucleic acids) which were perhaps included from the soluble supernatant fraction, as the particles could not be washed.

Nevertheless, the association of P.B.C. with cell-wall lipid is very interesting in view of the fact that the component responsible for the osmotic properties of the cell, the 'osmotic barrier' (Mitchell, 1949*a*), must necessarily lie close to the cell surface, and is widely believed to be lipid in character (e.g. see Work & Work, 1948). The fraction associated with P.B.C. has both of these properties. If, therefore, in its initial rapid reaction with the cell the penicillin molecule inactivates some component of the osmotic barrier, then the metabolic defects whose appearance coincides with the addition of penicillin should be closely related to osmotic barrier malfunction. Many such relations appear to exist in the literature, so that it is worth while to consider those surface changes which begin immediately the penicillin is added.

(1) *Effects of penicillin on the functions of an osmotic barrier.* Gale & Taylor (1947) showed that viability and active amino-acid transport across the osmotic barrier diminishes in parallel fashion in cells growing in presence of penicillin. Hotchkiss (1950), with different bacteria and growth conditions, found that only a little glutamic acid assimilation was prevented by penicillin but that polypeptides were liberated into the medium. This effect was confirmed by Gale & Paine (1951). However, as penicillin inhibits *Bacillus subtilis* in a medium containing only inorganic N (Hunter & Baker, 1949), the cell's disorganization is not limited to amino-acid uptake. A contrasting phenomenon, the effect of osmotic barrier destruction on penicillin uptake, is that loss of penicillin-binding capacity by intact cells and lipid particles proceeds simultaneously with cytolysis by a lipid-soluble substance such as phenol, whereas an anionic and a cationic detergent had no effect over the cytolytic range (Cooper, 1954).

(2) *Effect of penicillin on surface properties of the cell.* The cell surface is apparently synthesized *in situ* and is, therefore, either concerned in its own manufacture or is synthesized by the layer immediately underlying it, i.e. the lipid 'cytoplasmic membrane'. (There is some cytological evidence of cell wall synthesis by the cytoplasmic membrane, e.g. Bisset, 1950.) Thus it is relevant to this discussion that penicillin added to growing cultures immediately slows the formation of a polyhydroxy phosphoric ester (XSP) which comprises a large proportion of the cell wall of most penicillin-sensitive, but not resistant, organisms (Mitchell & Moyle, 1950). Since the cell surface increases, because the cell also swells, it follows that either the cell wall becomes very much more elastic, perhaps due to loss of XSP, or that a very considerable amount of other cell wall components are produced to make good the deficiency of XSP. It may be a reflexion of this change that the suspension simultaneously loses its stability (Mitchell & Moyle, 1951*a*) and that there occurs a progressive drop in surface charge which starts very soon after addition of penicillin (McQuillen, 1950–1). The very small number of penicillin molecules attached (Rowley *et al.* 1950) would themselves contribute a negligible amount to the charge on the bacterial surface. Streptomycin causes an opposite type of effect (McQuillen, 1951) in that there is an immediate change in surface charge on addition of this compound to resting cells, corresponding to a reversible adsorption of it, but there is no further effect on growing cells. The effect of penicillin in this case is, therefore, not common to all antibiotics, as is, for instance, the formation of 'giant forms'.

Another surface change reported is that a penicillin-resistant brucella organism lost the power to synthesize its surface capsule only when in presence of penicillin (Braun, Kraft, Mead & Goodlow, 1952). It is interesting that these surface changes presumably do not apply to the whole surface, as growth in penicillin does not affect the adsorption of phage on a sensitive staphylococcus (Elford, 1948).

(3) *Changes in properties of the strain on adaptation or selection to penicillin resistance or sensitivity.* When penicillinase production is not involved, changes in penicillin sensitivity are simultaneously accompanied by changes in many

properties. Among these are changes which appear to be related to osmotic or surface properties of the cells. For instance, highly resistant staphylococci lose ability to grow in 6·5% NaCl (Bellamy & Klimek, 1948; Klimek, Cavallito & Bailey, 1948), and resistant brucella mutants lose their smooth character (Braun *et al.* 1952). Gale (1947) found that an increase of, say, tenfold in penicillin resistance resulted in the glutamic acid assimilation system requiring tenfold the amount of penicillin to inhibit it, and the more resistant organisms were less able to assimilate glutamate (Gale, 1947). When relating changes in antibiotic sensitivities to the osmotic barrier, however, it must be remembered that any general change in cell permeability is likely to alter drug sensitivity.

In conclusion, the very small number of penicillin molecules bound by sensitive bacteria makes it seem probable that a very limited number of reactions, perhaps only one, may be governed by P.B.C. If this is so, the very diverse nature of the effects produced by penicillin in different organisms suggests that this reaction which is initially disorganized by addition of penicillin itself controls many diverse systems. The osmotic barrier in bacteria plays a vital part in the economy of the cell and it is shown above that there are many suggestions that the initial lesion caused by penicillin lies in the osmotic barrier. It is worth consideration, therefore, that the effects of penicillin in disorganizing, for example, nucleic acid metabolism (Mitchell, 1949*b*; Gale & Rodwell, 1948; Park & Johnson, 1949) may be due to the inability of the cell to assimilate, synthesize or retain some substance essential for such metabolism because of faulty functioning of the osmotic barrier.

I wish to thank Sir Alexander Fleming, F.R.S., for his continued interest in this work, and Dr D. Rowley for the help afforded by many useful discussions. Acknowledgements are due to the Central Research Fund, London University, for a grant for apparatus, and to Dr E. Lester Smith, of Glaxo Laboratories, for a generous gift of radiopenicillin G.

This paper is the second in the series 'The site of action of penicillin'.

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(*Received* 17 *August* 1953)

COOPER, P. D., CLOWES, R. C. & ROWLEY, D. (1954). *J. gen. Microbiol.* **10**, 246–249.

A Note on the Use of Radioactive Penicillin

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Use of radiopenicillin. One of the difficulties in work involving radioactive penicillin is the problem of ascertaining that the penicillin is free from radioactive impurities, which are constantly being produced owing to the instability of the penicillin molecule. The presence of such impurities gives incorrect specific activities and always allows the possibility that the uptake on the bacteria estimated as penicillin ^{35}S may, in fact, be due to these impurities. It is difficult to estimate the non-penicillin ^{35}S by paper chromatography since there is good evidence that some decomposition of the penicillin occurs on the paper (Lester Smith & Allison, 1952). The method which we have used latterly is that of counter-current distribution at room temperature, using *o*-methyl*cyclo*hexanone as the moving phase (Rowley, Steiner & Zimkin, 1946) and M-phosphate buffer (pH 5·4) as the stationary phase. The apparatus used was a 60-tube glass apparatus as described by Craig, Hausmann, Ahrens & Harfenist (1951). Under these conditions little, if any, decomposition of the penicillin can occur during the manipulations (Broderson, 1947) and at the end of the run the contents of each tube can be assayed for radioactivity and biological activity, allowing an accurate estimate of the impurities present. Fig. 1 shows the analyses of two samples of radiobenzylpenicillin. In both cases some non-penicillin radioactivity remained behind in the first few tubes, whereas the bulk of the penicillin was distributed in the tubes from 15 to 30. The mobility of the main peak was that to be expected of penicillin G (Sato, Barry & Craig, 1948) and corresponded exactly with the biological activity. The batch of old penicillin contained 15–20 % of radioactive impurities, whereas the crystalline material contained less than 5 %.

We have noticed that impure radiopenicillin gives a higher uptake curve on a sensitive strain of *Staphylococcus aureus* than crystalline or freshly extracted radiopenicillin. The pure material gives the plateau-like curve to be expected of an irreversible reaction with a component present in limited amount (for methods of measuring penicillin uptake see Rowley, Cooper, Roberts & Lester Smith, 1950). Uptake curves using materials similar to those described in Fig. 1 are shown in Fig. 2. It can be seen that, as is to be expected from the solvent insoluble nature of the impurities (Fig. 1), one extraction of the radiopenicillin into chloroform at pH 2·5 and back into buffer decreases the amount of impurities, and lowers the slope of the uptake curve's plateau almost to zero.

It is interesting that acid-degraded penicillin (largely penillic acid, Cook, 1949) gives a very high uptake curve (Fig. 2), while penicillinase-inactivated penicillin (penicilloic acid) gave a much lower uptake. It appears likely that the high adsorption of ^{35}S from old batches of radiopenicillin may be due to a mixture of these degradation products, which probably constitute the bulk of the radioactive impurities.

That the horizontal plateau of the uptake curve was due to penicillin, and penicillin only, was regarded as proved by: (*a*) the lack of effect on this portion of removal of all but $<5\%$ of radioactive impurities, as demonstrated by Craig distribution; (*b*) prevention of all uptake by pre-treating the cells with a small quantity (0·1 u./ml.) of crystalline ordinary penicillin; (*c*) the destruction of the horizontal portion, especially marked at low penicillin concentration, by inactivating the drug beforehand with the specific enzyme penicillinase (Fig. 2).

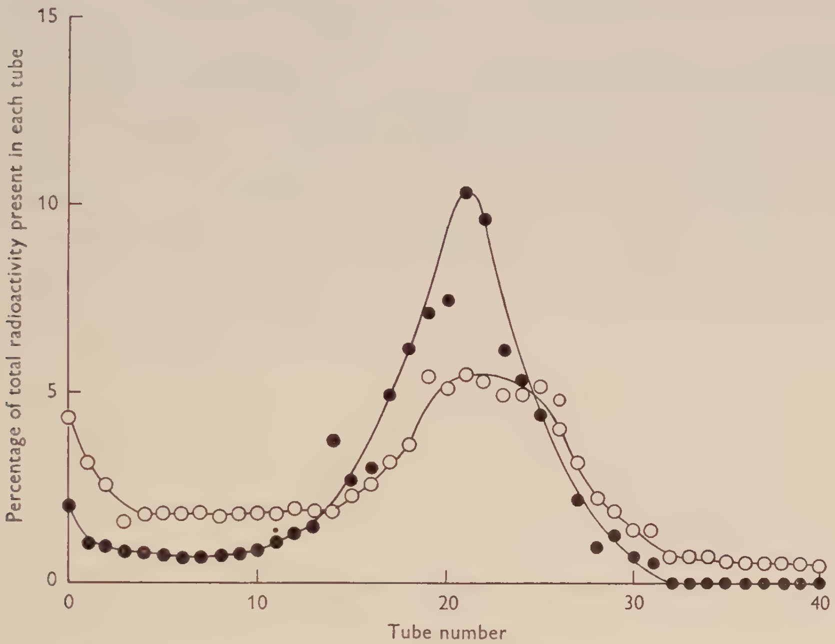

Fig. 1. Countercurrent distribution of ^{35}S from an old and impure batch of radiopenicillin (○—○) compared with a recently crystallized batch (●—●). The mobile phase was re-distilled *o*-methyl*cyclo*hexanone and the stationary phase was M-phosphate buffer (pH 5·4).

These changes in uptake curves during storage of radiopenicillin were found to give a more useful method of checking the purity of penicillin preparations than either biological assays of specific activity or the lengthy Craig analysis, and have been used routinely for several years. Therefore all batches of radiopenicillin after initial extraction from the culture medium were extracted into chloroform at pH 2·3 and back into neutral buffer. This extraction was repeated once. The radiopenicillin then contained not more than 5% of ^{35}S impurities by the Craig method and gave a nearly flat uptake curve on the standard organism. The specific activity was accurately determined by biological and radioactive assay, making due allowance for the non-penicillin ^{35}S. Henceforward the specific activity was read from a logarithmic decay curve calculated from the half-life of ^{35}S, and the purity of the material was checked periodically by an uptake curve on the standard organism. When the uptake plateau began to assume an upwards slope the material was re-extracted and re-assayed. The radiopenicillin was stored either in the dry state, when crystalline, or at pH 6·5 and 2° in 0·05 M-phosphate containing 1 mg. sodium hexametaphosphate/ml. as stabilizer. When the material was not crystalline no greater stability was obtained by freeze drying. Storing at −20° did not prevent production of ^{35}S impurities; this may be due to the pH changes which are likely to occur in the very high local concentration of salts just before freezing and after thawing. It is the experience of Lester Smith & Allison (1952) that penicillin is highly unstable in concentrated salt solutions.

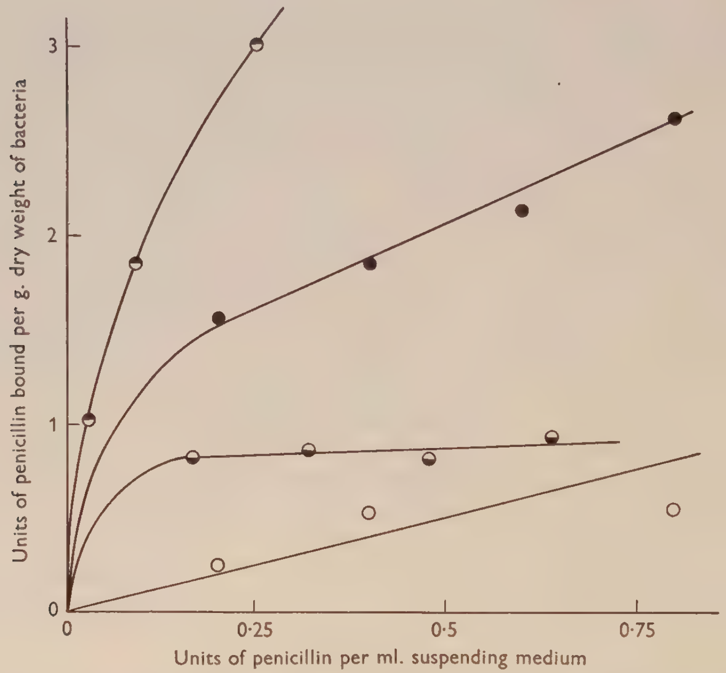

Fig. 2. Uptake of ^{35}S on a penicillin-sensitive staphylococcus using an impure batch of radiopenicillin (●—●); the same batch freshly extracted into chloroform at pH 2·3 and back into neutral buffer (◒--◒); freshly extracted radiopenicillin inactivated with penicillinase (sodium penicilloate, ○—○); radiopenicillin inactivated for 3 hr. at pH 2·5 and 37° and re-adjusted to pH 6·5 (◓—◓). 'Units of penicillin' in the ordinates refer to penicillin or its molar equivalent as degradation products.

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(*Received* 17 *August* 1953)

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The Purification and Electron Microscopical Examination of the Structure of Staphylococcal Bacteriophage K

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SUMMARY: Staphylococcal bacteriophage K was concentrated and purified from broth lysates without the use of high-speed centrifugation, which inactivates this phage. After precipitation with 30% (v/v) acetone in water in the cold, the phage was further purified by treatment with *iso*butanol and chloroform, and concentrated by pressure filtration in reinforced collodion sacs. Electron microscopical examination revealed the presence of empty phage heads and tails, in addition to intact phage in the purified concentrate. Experiments indicate that while these entities could be made by disintegration of the phage, they were already present in this form in the original lysate. Study of the structure of the phage indicates the presence of a removable solid rod or plug within the tail which terminates in the terminal bob. These findings are discussed in relation to the structure of other phages, and their physiology.

During attempts to concentrate and purify the staphylococcal phage K, the appearance of the product was frequently examined by electron microscopy; particles were seen which appeared to be 'pieces' of the phage. This phage is a tailed variety and the pieces seen appeared to correspond to individual structures, such as loose tails, of which the intact phage was composed. A method of purification was devised which did not result in the fractionation of these structures until the final stage, while still permitting the concentration of the phage and removal of contaminating particulate debris of about the same size as the phage. This method is described below with the results of electron microscopical examination of the products.

METHODS

Organism. The organism used throughout was the Oxford strain of *Staphylococcus aureus,* NCTC 6571. The phage used was staphylococcal bacteriophage K. Hartley tryptic digest broth was used as nutrient medium throughout.

Bacteriophage assay. This was made by plaque counts on the surface of 1% nutrient agar plates.

Electron microscopy. Examinations were made with a Siemens electron microscope. Each specimen was prepared at several dilutions in distilled water containing 0·05% osmic acid; a drop of each dilution was placed on a collodion covered mounting grid and allowed to dry over phosphorus pentoxide. The material was shadow cast before being photographed in the microscope: gold-manganin approx. 6 A. thick at an angle of 15° was used for shadowing.

The assessment of the efficiency of the purification procedures used. Since the bacteriophage was being prepared mainly for electron microscopical study, examination of the product by this method was made at each stage of the purification process. In addition, chemical estimations were made of the nitrogen and in some cases the phosphorus content of the preparation. As a result of this, the opinion was formed that an electron micrograph made by the technique described gave excellent qualitative information on the purity of the product. The dried material used for microscopy contained all the constituents present in the preparation; thus, crystalline or amorphous deposits from dissolved impurities and particulate matter were readily seen. Small quantities of other dissolved impurities, such as proteins, caused a diminution in sharpness of the outlines of phage particles, while larger amounts completely obliterated them. It was found that chemical estimations frequently gave less useful information than electron microscopical examination; consequently more importance was attached to the latter method. This was particularly true for procedures designed to remove particulate material of the same order of size as the bacteriophage.

The production of bacteriophage. Cultures were grown either in 4 l. bottles (containing 1 l. of broth) aerated by mechanical shaking, or in larger vessels aerated by air forced through a porcelain filter candle which was immersed at the bottom of the medium; the porosity of the candle was not critical. Foaming was controlled by small quantities of tributyl citrate or silicone antifoam. The ratio of initial phage particles added to the number of bacterial cells present was found to be unimportant so long as sufficient phage was added during the growth of the host cells to cause lysis to be complete shortly before growth ceased in a control culture without phage. A typical infective ratio used was an initial concentration of approximately 10^6 phage particles and 3×10^8 viable cells/ml. (determined by colony counts). Such a system gave complete lysis in 3–4 hr. and contained $1–2 \times 10^{10}$ plaque-forming units/ml.

The concentration of bacteriophage. Centrifugation in the Sharples centrifuge was found to cause inactivation of this phage. Other methods of concentration tried included adsorption, elution, vacuum distillation, enzymatic digestion, precipitation and pressure filtration. The last two of these were found to be the most useful. Several reagents, including acetone, methanol and ethanol, and ammonium sulphate were used as precipitating agents. Preliminary tests indicated that the phage was most stable to acetone and this was chosen for more detailed study, the results of which are shown in Table 1. It was evident that the phage was able to withstand acetone precipitation for 18 hr. at 0°. Thirty to thirty-five % (v/v) acetone in water was used to concentrate the phage in larger volumes of lysate. By using acetone at $-10°$ and the lysate at 0°, from 50 to 100 % of the viable phage was recoverable in an aqueous solution of the precipitate. This solution was turbid and contained from one-tenth to one-twentieth of the nitrogen originally present in the lysate. In view of the surprising stability of this bacteriophage to dilution in pure acetone (see Table 1), an attempt was made to find a two-phase system, in which the bacteriophage might partition in a constant manner, enabling a purification to be effected by either a countercurrent technique (Craig, 1949) or by partition chromatography on some supporting column. Such systems have been developed by Martin & Porter (1951) with remarkable results for the separation of enzymes. However, no system was found in which the bacteriophage would partition between both phases.

Table 1. *The stability of staphylococcal phage K to acetone at 0° for 18 hr.*

All tubes were centrifuged at 18 hr., and the pellets resuspended in 10 ml. broth for titration. Cold acetone was added to the cold mixture of 0·1 ml. of diluted phage, and broth.

Tube no.	Broth (ml.)	Acetone (ml.)	1st expt., plaque count of supernatant	Plaque count of pellets	2nd expt., plaque count of supernatant	Plaque count of pellets
1	10	—	8	1	8000+	75
2	9	1	19	1	8000+	80
3	8	2	16	2	8000+	378
4	7	3	7	5	800	8000
5	6	4	1	6	92	6000
6	5	5	1	11	92	4000
7	4	6	1	4	60	5000
8	3	7	—	4	37	3000
9	2	8	—	6	11	3000
10	1	9	—	2	—	23
11	—	10	4	—	2500	10

During tests with *iso*butanol, and to a lesser extent with chloroform, it was noticed that a very stable emulsion was frequently formed on shaking the solvents with aqueous suspensions of the acetone precipitate of phage lysates. When the system was centrifuged at low speed, the two phases were separated, with debris and a small amount of emulsion remaining at the liquid/liquid interface. The preparation which had been originally white and opaque was now quite translucent, and showed a strong bluish opalescence; titrations of the aqueous phase showed it to contain 90 % or more of the original phage. The change in nitrogen content of the preparation as a result of this treatment was measured and found to be about halved; it seemed likely that bacterial debris was being retained in the emulsion; this was confirmed by electron microscopy (see below). In one experiment, treatment with *iso*butanol and chloroform reduced the optical density (using a tungsten lamp without a filter) of the preparation from 0·63 to 0·11 without change in titre.

Treatment of the acetone precipitate of a phage lysate in this manner with *iso*butanol was considered to be of great value in removing from the phage preparation particulate material which was very difficult to separate by other methods. Under optimal conditions about 95 % recovery of bacteriophage could be obtained without washing the organic phase emulsion for residual phage; the only suspending medium for the phage, which gave this recovery, was distilled water. Broth suspensions lost up to 90 % of their phage content; saline and buffer solutions gave intermediate values. The bacteriophage was progressively inactivated in water, losing about 90 % of activity at 0° in 1 week. Therefore, it was only kept in aqueous suspension for a few hours. In practice, an *iso*butanol + chloroform mixture is more convenient to use than *iso*butanol alone. Chloroform kills bacterial contaminants of the suspension, and the density of a 50/50 mixture of these solvents is greater than that of water, thus facilitating removal of the upper aqueous phase after centrifugation. Maximal yields of bacteriophage were only obtained with phage titres below 10^{11}/ml.; higher concentrations gave progressively lower yields.

Concentration of the aqueous bacteriophage suspensions. After the above treatments, the resulting suspension of phage was still relatively dilute and contained dissolved impurities. A simple method of concentration was found to be pressure filtration at 0° through reinforced collodion sacs prepared by a method developed by van den Ende, Don, Elford, Challice, Dawson & Hotchin (1952). Sacs prepared in this way withstand a pressure of 20 lb./sq.in., which is sufficient to concentrate about 500 ml. of phage suspension/sac/24 hr., using a reservoir of the dilute phage suspension fed under pressure into the sac. In this process a variable amount of soluble impurity is lost, depending on the porosity of the sac. The recovery, when using this concentration method performed in the cold, was very good, 80–95 % of infectivity being regularly obtained. Final purification of the bacteriophage concentrate to remove remaining soluble material was obtained by centrifugation at about 10,000 g.

The purification procedure finally adopted can be summarized as follows. The crude lysate was collected as soon as lysis was complete and stored overnight at 0°. Acetone at −10° to −15° was added at 0° with stirring to give a final concentration of 30 % (v/v). When precipitation was not obvious, the concentration of acetone was increased to 35 %. After standing for 2 hr. at 0° the precipitate settled and the clear supernatant was poured off. The lower layer was centrifuged at 0° for 5 min. at low speed to sediment the precipitate. The latter was dissolved in distilled water to give a final concentration of about 5×10^{10} phage particles/ml. and shaken for 5 min. at 0° with half its volume of a mixture of equal parts of *iso*butanol and chloroform. The resulting emulsion was centrifuged at low speed to separate the two phases, the upper aqueous one (containing the phage) then being removed. The *iso*-butanol + chloroform treatment was repeated twice. The aqueous suspension was then concentrated by pressure filtration through collodion sacs to a few ml. The preparation at this stage (concentrate) was found to contain the formed elements described in the next section and also a variable amount of protein and other impurities in solution. The phage could be removed and washed by centrifugation at about 10,000 g without loss of activity; final suspension was made in saline or in water for electron microscopy. Details of the yields obtained in this purification procedure are given in Table 2. The purified phage had a nitrogen content of 7×10^{-16} g./plaque-forming unit. The nitrogen : phosphorus ratio was 5·0 g./g.

Table 2. *Yield of plaque-forming units obtained during purification*

Stage of purification	Yield as % of previous stage
Acetone precipitation	50–100
*iso*Butanol + chloroform treatment	85–95
Pressure filtration	80–95
Centrifugation at 10,000 g	80–90
Washing and recentrifugation	70–90
Overall yield	19–73

RESULTS OF ELECTRON MICROSCOPICAL EXAMINATIONS

The bacteriophage concentrate obtained as described above was found to contain what appeared in electron micrographs to be intact phage particles, loose phage tails and empty heads without tails. After centrifugation at 10,000 r.p.m. for 2 hr. in an Ecco centrifuge with swing-out buckets, three layers were visible in the preparation. These consisted of a slightly opalescent supernatant or top layer, a very opalescent middle layer, and a translucent amber pellet. Samples of each of these layers were removed and examined electron microscopically. The bottom layer, or pellet (Pl. 1, fig. 1), consisted almost entirely of intact phage particles; the middle layer (Pl. 1, fig. 2) consisted largely of what appeared to be empty phage head membranes. The top layer (Pl. 1, fig. 3) contained some of the structures seen in the middle layer and, in addition, what appeared to be separate tails. Pl. 1, fig. 4, shows the state of the preparation before the *iso*butanol treatment. The phage is seen to be contaminated by the presence of circular 'foamy' bodies, an intact staphylococcus, some small particles, and amorphous debris. There are present some free head membranes as described above and a few tails. The decrease in turbidity observed after treatment of this stage with *iso*butanol is apparently due to the removal of the 'foamy' structures and remaining unlysed organisms seen in Pl. 1, fig. 4.

The origin of separate phage head membranes and tails. It was thought at first that if the structures described in Pl. 1, figs. 2 and 3, are in fact head membranes and tails, they might have been produced by the purification procedure. However, this is shown not to be the case by the following facts: (i) similar structures are seen in Pl. 1, fig. 4, before *iso*butanol treatment; (ii) repeated *iso*butanol treatment of the pure phage (as shown in Pl. 1, fig. 1) neither significantly altered its plaque-forming titre, nor produced an appreciable number of head membranes or tails in subsequent electron micrographs; (iii) electron microscopy of the original lysate prepared by adsorbing the lysate constituents on to the collodion membrane (pictures were too dense when prepared by the standard procedure) showed the presence of head membranes and tails; (iv) similar structures have been described by other workers (see Discussion) in preparations of this and other phages prepared by different techniques.

The production of head membranes and tails from intact phage. In order to demonstrate that the head membranes and tails were the same as corresponding structures in the intact phage particle, an attempt was made to break down the purified phage into free tails and empty head membranes, plus the contents of the heads. Such a breakdown was achieved by shaking 5 ml. of a suspension of the purified phage preparation (containing about 5×10^{10} plaque-forming units/ml.) for 2 hr. in a Mickle (1948) disintegrator without the addition of any abrasive; under these conditions the titre fell by a factor of about 100. Plate 1, fig. 5, shows an electron micrograph of the purified bacteriophage (seen in Pl. 1, fig. 1) after 2 hr. shaking in the Mickle disintegrator. It can be seen that very little normal phage remains; free phage tails are present, and free empty head membranes, many of which are clumped together. In addition, there is some fibrillar material and a few small particles. Several of the tails appear to show evidence of internal structure; this will be discussed later. After disintegration by shaking, the phage preparation was centrifuged under the same conditions as those required to separate the whole phage, head membranes, and tails into three layers, as described above. The total amount of material used was small, and the preparation after centrifugation could only be divided into a small pellet and a clear supernatant. Pl. 2, fig. 6, shows an electron micrograph of the pellet, and Pl. 2, fig. 7, shows the supernatant. It can be seen that the appearance and behaviour of the structures obtained by disintegration of the phage are very similar to the head membranes and tails extracted from the lysates. It was concluded that they are essentially the same.

The appearance of osmotically shocked K bacteriophage. A portion of the purified bacteriophage (the 'bottom-pellet' of the phage concentrate) was subjected to osmotic shock as described by Herriott (1951). Its titre fell by a factor of about 1000. The appearance of the preparation following shocking is seen in Pl. 2, fig. 8; empty head membranes are seen, also free tails and some complete phage units with empty heads. There is a large amount of fibrillar substance which is dotted with small particles, with diameters somewhat less than that of the phage tail. This preparation was deproteinized by shaking with a mixture of octyl alcohol and chloroform and was examined spectrophotometrically. A nucleic acid type of absorption spectrum was obtained with a minimum at 2370 A. and a maximum at 2560 A.

Conclusions as to the structure of the K bacteriophage

During the examination of many electron micrographs obtained during the experiments described above, particularly in the photographs of disintegrated phage, many examples were obtained which appeared to show more structural detail than has been described for this or any other phage. The K bacteriophage was described by Smiles, Welch & Elford (1948) as consisting of a spherical head and a relatively long tail which terminated in a round 'bob'. This description is confirmed by the present study; in addition, evidence is presented which indicates that the tail of this phage consists of a hollow cylindrical sheath, containing a central solid rod, which terminates in the tail bob at one end and inside the nucleic acid-containing head at the other. Examples of photographs suggesting this are shown in Pl. 1, fig. 5, and Pl. 2, fig. 7. Countless other examples have been found, some of the most typical of which are shown in Pl. 2, figs. 9–14; figs. 9–13 are from Mickle disintegrated phage and the remainder from various batches of partially purified phage. Pl. 2, fig. 9, shows an intact free tail; in fig. 10 can be seen two tails, each of which is composed of a thick and thin half; in fig. 11 can be seen other examples of a constriction or narrowing of the tail, and in fig. 12 the tail is separated from the head by a 'neck' consisting of a narrow rod or thread. Pl. 2, figs. 13 (top) and 14, show examples of what is considered to be the terminal bob of the tail, attached to a relatively thin rod which has become detached from the tail of a phage particle. The size of the rod fits the assumption that it is the same structure as seen projecting from portions of tails in other pictures, and that it is the normal internal core of the phage tail. The structure of the phage, as indicated by these experiments, is indicated diagrammatically in Fig. 1. Preliminary chemical analysis indicates that the head membrane and tail are composed of protein with little, if any, phosphorus present; no detailed analysis of the composition of these structures has been made.

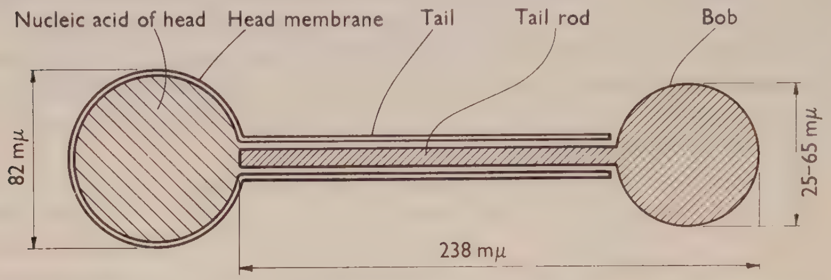

Fig. 1. Diagram showing the possible structure of staphylococcal bacteriophage K as indicated by electron microscopy.

DISCUSSION

Many different techniques have been used in the past to purify and concentrate bacteriophage. Clifton (1930) used adsorption on to alumina to purify staphylococcal phage, and this method has been expanded by Wahl *et al.* (1939, 1950) and others. Concentration on collodion membranes was used by Elford (1938) and by Hershey, Kalmanson & Bronfenbrenner (1943), and concentration on foam was used by Bloch (1940). Northrop (1938) used several original methods in a study of the concentration and purification of a staphylococcal phage; his methods included low temperature distillation *in vacuo*, digestion with crystalline trypsin, and ammonium sulphate and lead acetate precipitation of the bacteriophage. Organic solvents have been tried as precipitating agents by Bronfenbrenner & Korb (1925) and by Wahl & Blum-Emerique (1949) who obtained good results with ethanol. Acetone was tested by Colwell (1937) and found to inactivate coli phage, but this agent did not destroy staphylococcal phage K at low concentrations in experiments performed by the late W. J. Elford (personal communication). Most of the above methods were tested for applicability to the concentration and purification of staphylococcal phage K in the course of the work described in this paper.

The purification method finally adopted and described here represents the most efficient procedure of those tested for this phage, obtained in a particular way. It is of some interest to compare the values for the nitrogen content of the phage with those of Northrop (1938), who worked with a staphylococcal phage, and found a sevenfold lower nitrogen content for his phage (1×10^{-16} g. N/infective unit) than that found in the present work. Whether or not the two phages are identical is not known, nor is the proportion of viable K phage particles in the preparation; estimations of the opalescence of some of the pure preparations of identical titre indicate that this was very variable, and sometimes quite low. This variability seemed to be related to the proportion of viable phage particles due to changes in the conditions of bacteriophage production, rather than purification; it was not investigated further.

A method of virus purification which largely avoids high-speed centrifugation has several merits. It is very suitable for working with large volumes of material; it may be useful for small viruses less easily concentrated by centrifugation and is essential if the virus is inactivated by the usual methods of high-speed centrifugation. The finding that staphylococcal phage K is susceptible to high centrifugal stress confirms a report by Wyckoff (1938). A method of purification of T_2 bacteriophage similar to the present one has recently been devised by Herriott & Barlow (1952); these workers concentrated and purified the bacteriophage by precipitation at a lowered pH.

From the study described here, it is evident that centrifugal methods of purification of staphylococcal bacteriophage K result in removal from the final product of the head membranes and free tails described. It is possible that similar structures to those described here will be found for other phages if methods of concentration are used which do not exclude them. There is evidence (see below) that this is so for T_2 phage when purified by the method of Herriott & Barlow. The treatment with *iso*butanol and chloroform, although arrived at in quite a different way, appears to operate in an analogous manner to the deproteinization of solutions by shaking with chloroform described by Sevag (1934). In both cases, the impurity collects and is removed at the interfacial surface between the organic and aqueous phases. The bacteriophage is presumably protected from surface inactivation by the slight excess of protein remaining in solution during the treatment; the substitution of octyl alcohol (commonly added to chloroform as an anti-foam agent) for *iso*butanol caused a marked loss of bacteriophage. The 'foamy structures' removed by *iso*butanol and chloroform treatment are thought to be remnants of the cell membranes of lysed cells. The stability of the phage in almost 100% acetone (Table 1) seemed most remarkable. Although this observation led to the use of other organic solvents, no other practical use has so far been made of it.

The value of electron microscopical examination in assessing the purity of phage preparations may be stressed. Ideally, quantitative electron microscopy as described by Williams & Backus (1949) and by Backus & Williams (1950) is required before chemical analysis can be equated with the number of phage particles. The plaque count will then show the proportion of these which are viable.

Preparations of bacteriophage containing empty head membranes and tails were reported by Smiles *et al.* (1948) and by van den Ende *et al.* (1952). Heden (1951) and Herriott & Barlow (1952) reported small 'phage-like' bodies in T_2r+ lysates. These bodies have a small head and a thinner tail than the T_2 phage, but are about the same length as the tail. They appear to be associated with infection by the T_2r+ phage, and may correspond to the 'rod and bob' unit described in this paper and shown in Pl. 2, figs. 13 and 14.

The results of Kellenberger & Kellenberger (1952) are of extreme interest in connexion with the rod units reported here. These workers published electron micrographs of a most unusual group of bacteriophages obtained by ultraviolet-induced lysis of *Bacillus cereus*. Photographs of some of these phages show the head of the phage to be separated from the tail by a 'neck', and are strikingly similar in appearance to Pl. 2, fig. 12. Other variations are shown, closely resembling those described in this paper. It appears that one of the *B. cereus* phages normally has near the head a portion of its tail constricted as a 'neck'; the authors considered that some of the other forms they found were derived from this. There is apparently some indication that the tail structure described here may be of general occurrence among bacteriophages.

The pitfalls of a too ready acceptance of electron microscopic appearances are becoming well known. If it be accepted that the phage tail, at least in some cases, contains an inner rod-like core which is readily separable from its outer sheath, it is tempting to speculate as to its function. Herchey & Chase's results (1952) on the deoxyribosenucleic acid injecting action of the T_2 bacteriophage have revolutionized the conception of phage infection. In Hershey's results only about 80 % of the phage protein is accounted for outside the host after infection. This leaves room for the speculation that the inner core or rod of the tail may enter the bacterium before the nucleic acid. If this should be so, it is not inconceivable that this part of the tail may be responsible for the inhibition of host cell function. This property has been shown by Herriott (1951) to reside in nucleic acid-free osmotically shocked 'ghosts' of T_2 phage. The significance of the small spherical particles seen in several of the present photographs, especially Pl. 2, figs. 7 and 8, is not known. It is felt that these may be artifacts due to fixation or drying, as their presence and appearance has been very variable. However, they may be present as particles within the phage head, and the appearance of the phage head in Pl. 1, fig. 1, does suggest this, although this again may be an artifact due to the drying technique used.

I wish to acknowledge the excellent technical assistance of Mr D. V. Law and Mrs I. M. Honey who made most of the electron micrographs.

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EXPLANATION OF PLATES

For detailed description of figures, see text.

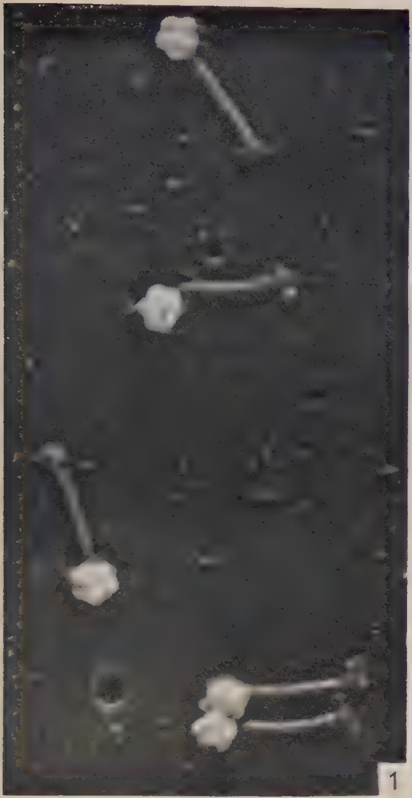
PLATE 1

Fig. 1. Electron micrograph of staphylococcal bacteriophage K. × 67,650.
Fig. 2. Empty heads of staph. bacteriophage K. × 23,250.
Fig. 3. Tails of staph. bacteriophage K. × 48,960.
Fig. 4. Partially purified phage preparation. × 12,240.
Fig. 5. Purified bacteriophage after treatment in Mickle disintegrator. × 28,980.

PLATE 2

Fig. 6. Preparation of head membranes from disintegrated phage. × 25,135.
Fig. 7. Preparation of tails from disintegrated phage. × 38,480.
Fig. 8. Appearance of staph. phage K after osmotic shock. × 36,080.
Fig. 9. Typical free phage tail. × 52,580.
Fig. 10. Phage tails showing details of internal structure. × 52,910.
Fig. 11. Phage tails showing constricted portions. × 46,970.
Fig. 12. Phage particle showing the head separated from the tail by a 'neck'. × 48,510.
Fig. 13. Portions of phage tail, including inner rod, or 'plug', and terminal bob. × 38,240.
Fig. 14. Portions of phage tail, including inner rod, or 'plug', and terminal bob. × 46,970.

(*Received* 20 *August* 1953)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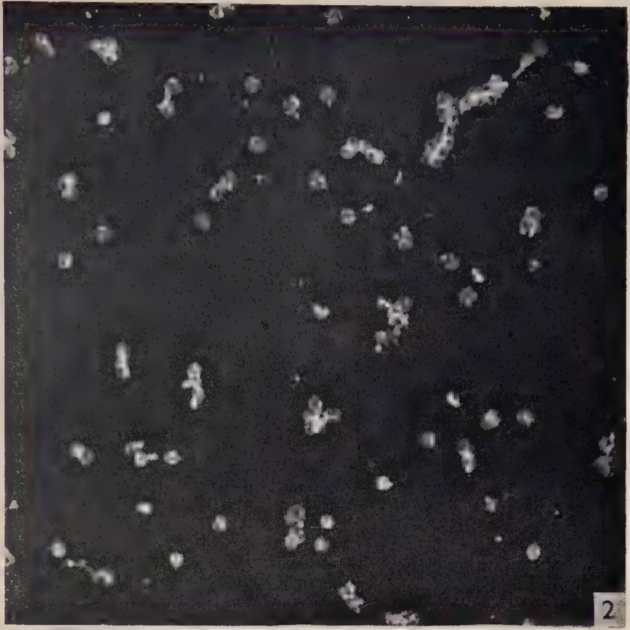
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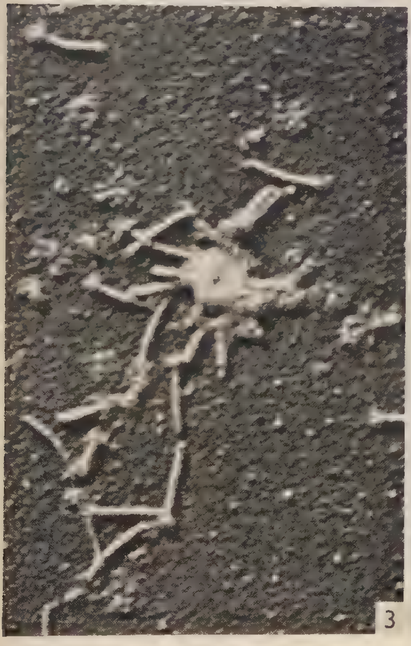
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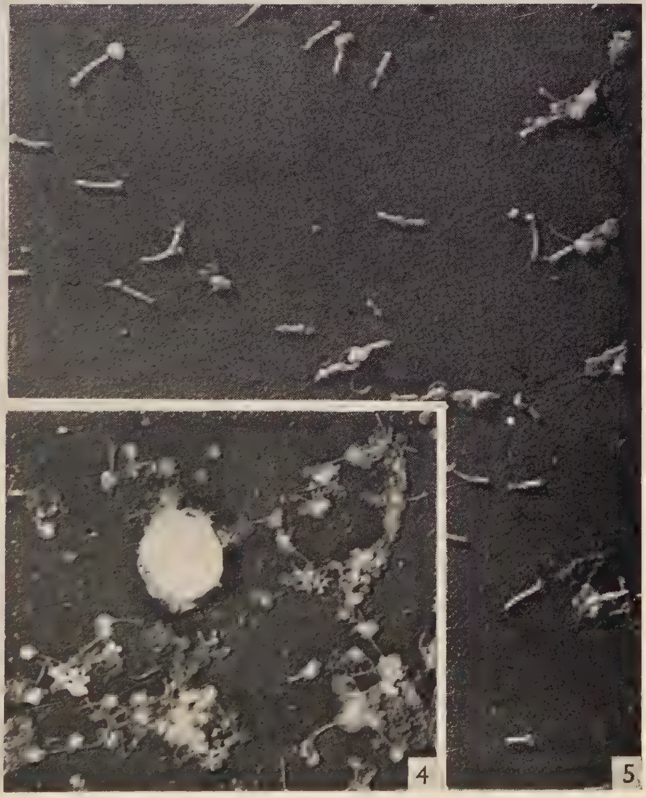
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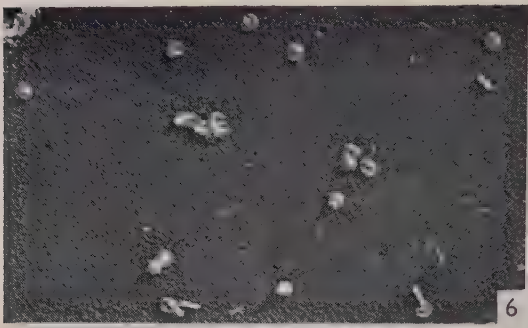
J. E. HOTCHIN—STAPHYLOCOCCAL BACTERIOPHAGE K. PLATE 1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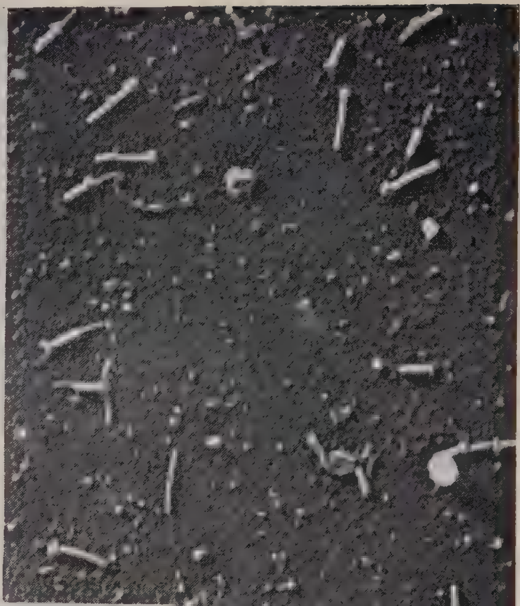
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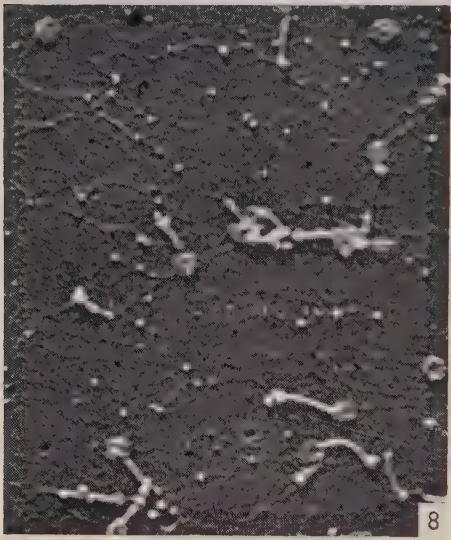
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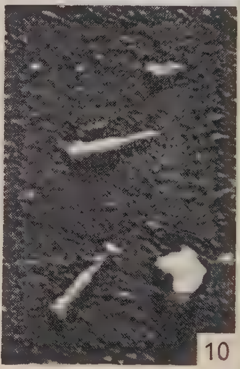
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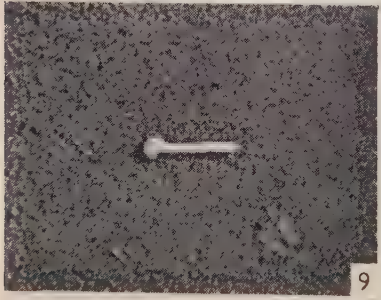
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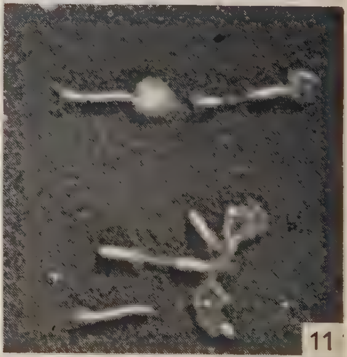
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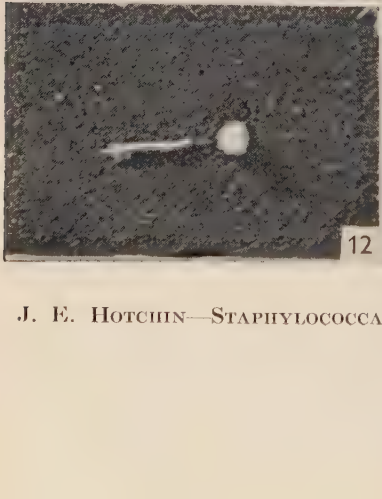
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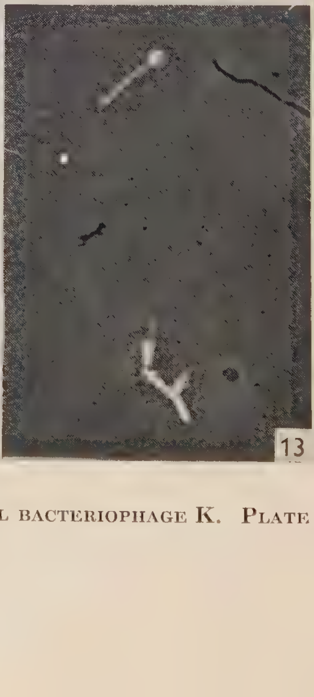
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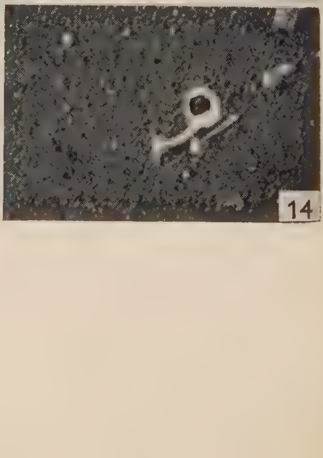
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J. E. Hotchin—Staphylococcal bacteriophage K. Plate 2

Happold, F. C., Johnstone, K. I., Rogers, H. J. & Youatt, J. B. (1954). *J. gen. Microbiol.* **10**, 261–266.

The Isolation and Characteristics of an Organism Oxidizing Thiocyanate

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SUMMARY: The isolation, morphology, cultural characteristics and growth requirements of an autotroph which oxidizes thiocyanate are described. It is suggested that the organism be assigned to the genus *Thiobacillus*.

The oxidation of thiocyanate by bacteria was described by Happold & Key (1937); it was not then realized that the organism was an autotroph until it was isolated in pure culture (Happold, Johnstone & Rogers, 1952). The present paper contains a fuller description of the organism. The literature will be more comprehensively reviewed in a later paper on the metabolism of the organism.

Isolation of the organism

Material for isolation was obtained from sewage effluents and also from a well water which contained thiocyanate. The cultures were enriched by percolation, in the presence of thiocyanate, through columns designed to approximate to the conditions obtaining in filter beds. The final isolation was made by the selection of single organisms, but it was first necessary to decrease the numbers of contaminating heterotrophs. The crude cultures, although maintained in a very simple medium, appeared from plate counts for heterotrophic organisms to contain heterotrophs in numbers exceeding those of the autotroph. The basal medium used for growth of the thiocyanate organisms was: Na_2HPO_4, 1·0 g.; KH_2PO_4, 0·6 g.; KCNS, 0·2 g.; distilled water to 1 l. The pH of the medium was 7·0–7·2. Contaminating heterotrophs were able to grow in these cultures even when the medium was prepared from water twice distilled from alkaline potassium permanganate and analytical grade reagents. Glassware was cleaned with aqua regia, and cotton-wool plugs were replaced by inverted glass beakers.

Selection of colonies from a solid medium, prepared by addition of agar to the basal medium, failed to give any cultures which oxidized thiocyanate. Flooding of the plates with ferric nitrate reagent showed that removal of thiocyanate had rarely occurred other than in areas of confluent growth. These experiments suggested that the agar, or some contaminating material, might have an inhibitory effect on the autotroph. It therefore seemed necessary to eliminate agar from the medium and to use silica gel in its place.

Silica gels were prepared by a modification of Taylor's method (Taylor, 1950) obtained from Dr W. K. Smith (Butterwick Research Laboratories, Welwyn; private communication). A 10% (w/v) solution of sodium silicate was passed through a column of Amberlite 120 exchange resin. The resulting sol was stable to autoclaving and could be gelled by the addition of sterile solutions of the required salts. The amounts of salts usually added to 1 l. of the basal medium were dissolved in 125 ml. distilled water, autoclaved (15 lb./sq.in. for 15 min.) and added to the sterile silicate solution in the proportion of 1 vol. to 3 vol. of silicate solution.

Addition to the medium of some compound which might act as an indicator of the autotrophic colonies was tried without success. There was no great pH change during oxidation of thiocyanate, and no indicator for the presence of thiocyanate or sulphate could be used without inhibiting growth. However, it was found that when two plates were prepared, differing only in the presence of bromthymol blue in one, and were inoculated and incubated at 22° for 3–4 days, a colony form was missing from the plate containing indicator which was present on the other. This colony type was selected. Subsequent flooding of the plates with ferric nitrate reagent indicated that oxidation of thiocyanate had occurred on this plate and not on the plate containing the indicator. A culture was thus obtained which oxidized thiocyanate and would not grow, for example, on ordinary nutrient (peptone-containing) media.

Such a culture was used for the isolation of single organisms. The organisms were grown in the defined liquid medium containing thiocyanate at 22° until a faint turbidity appeared. A droplet of this culture was placed on the surface of a plain 2·5 % agar gel and single organisms were isolated, using dark ground illumination (Goldie, Gordon & Johnstone, 1948; Johnstone, 1953). Eight individual organisms thus isolated were separately transferred on small cubes of agar gel to shallow layers of sterile thiocyanate medium in conical flasks, and incubated at 22°. All the eight organisms were viable and utilized thiocyanate. One strain was adopted for further work. Although agar media were inhibitory to the organism, it was possible to use agar for the single cell isolation because very small blocks of agar were added to the liquid medium and the organism then grew in liquid culture.

Morphology

The organism, as may be seen from the electron micrograph (Pl. 1), is a parallel-sided, round-ended rod 0·5–1·5 μ. long and *c.* 0·3 μ. wide, with a single polar flagellum. The halo-like effect has been seen to a greater or less extent on all the pictures examined. The organisms are Gram-negative, and we have seen no evidence of spore formation.

The organism is actively motile when observed by dark-ground illumination using a drop of fluid culture placed on the surface of agar or on a glass slide. Motility has been observed in freshly isolated cultures, and after 18 months of laboratory cultivation. In addition to the usual straight swimming across the field, movement in circles, both clockwise and anti-clockwise, has been observed. Some organisms rotate rapidly without progression. Finally, other organisms appear to attach themselves to the surface of glass or agar and continue to move the free end of the rod, thus describing a cone of rotation. This type of motility is said to be characteristic of polar-flagellated organisms.

Cultural characters

In a basal liquid medium incubated aerobically at 30°, growth appears after 24–48 hr., depending on size and age of the inoculum. After the disappearance of the thiocyanate there is a marked tendency to clumping. In older cultures the clumped material was found to contain elementary sulphur; the addition of fresh thiocyanate to a culture which has been stored without thiocyanate for some time will often result in the separation of free sulphur. Young cultures, however, oxidize thiocyanate quantitatively to sulphate. Table 1 shows the production of ammonia and sulphate from thiocyanate. Thiocyanate was estimated colorimetrically with ferric nitrate reagent, ammonia with Nessler's reagent and sulphate turbidimetrically as $BaSO_4$ by the addition of solid $BaCl_2$ and comparison with freshly prepared standards. A Unicam D.G. spectrophotometer was used for the three estimations. The culture was incubated at 30° and samples were withdrawn at intervals. The Table shows the disappearance of thiocyanate, the appearance of ammonia and sulphate and the calculated theoretical amounts of each.

Table 1. *The production of ammonia and sulphate from thiocyanate by* Thiobacillus thiocyanoxidans *expressed in* μg./ml.

Time (hr.)	KCNS loss	NH_3 as N found	NH_3 as N theor.	$SO_4^{=}$ as S found	$SO_4^{=}$ as S theor.
0	0	0·1	0	0	0
42	15	0·55	2·1	2	4·93
65	20	1·45	2·9	5	6·6
72	30	2·7	4·2	7	9·9
89	55	6·4	7·9	17	18·1
96	82	9·8	11·8	24·5	27·2
161	210	27·5	30	65	69·3
168	216	30	31	70	71·2
185	234	33	33·7	77·5	77·8
192	240	33	34·3	77·5	79·1

Samples were withdrawn at the times stated from a liquid medium containing KCNS (240 μg./ml.) and phosphate (380 μg./ml.) incubated at 30°.

Reference has already been made to the difficulty of growing the organism on the defined medium solidified with agar. That the organism can grow on agar-containing media has been clearly demonstrated (Happold & Key, 1937; Happold *et al.* 1952) and it seems probable that the organism can be trained to do this. A culture was obtained by prolonged incubation on a mineral salts agar. The first growth was slow, but a subculture was found to grow and oxidize thiocyanate in 3–4 days. Some months later, when none of the original sample of agar was available, an attempt was made to grow the organism on agar samples obtained from different sources. On all of them growth then took some weeks to become visible. One sample of agar was washed by allowing plates to stand in large volumes of distilled water. When the medium prepared with this washed agar was inoculated, the rate of growth increased in successive subcultures and then maintained a growth time of three days at 30°.

On thiocyanate mineral agar, the colonies were pin-point in size, circular, with entire margins and convex. In early stages they were translucent and bluish but on further incubation they became opaque and yellowish. This latter effect seemed possibly to be connected with the observed production of free sulphur in liquid cultures.

The colony form on the silica gel medium showed considerable variation, depending chiefly on the degree of moisture in the gel. The colonies were usually larger than those on agar and were flat. When the organism was first isolated, the colonies grown on silica gel were circular with entire margins and, at first, translucent, but on further incubation the outer rim of the colony became opaque. After laboratory cultivation of the strain, these types of colony were not seen and they appeared uniformly opaque on ageing. Colony size on silica gel varied from pin-point on dry surfaces to large spreading colonies on wet plates; the average colony diameter was *c.* 0·5 mm.

Growth requirements

The organism did not grow on the usual laboratory media such as peptone and yeast extract, casein digests or nutrient broth. No organic compounds have been found which will support growth in the absence of thiocyanate or improve growth in its presence. Compounds which have been tested at various concentrations include: peptone (Difco), yeast extract (Difco), glutamic acid, lactate, pyruvate, acetate, citrate, fumarate, succinate, glucose and phenol. Phenol was tested because the organism grows in its presence in gas-works liquors. Growth always occurred at normal rates in low concentrations of the organic compounds. Glutamic acid was completely inhibitory at 0·02 % (w/v). The other compounds showed some variation, but all were inhibitory at 1 % (w/v) and most showed considerable inhibition at 0·1 %. In the presence of organic compounds no growth occurred without the oxidation of thiocyanate.

The organism required an oxidizable form of inorganic sulphur: thiocyanate, thiosulphate and sulphur were utilized. It seems likely, from the results of study of respiratory metabolism, that sulphide is also suitable and growth with sulphide has been observed, but owing to the production of traces of sulphur, some uncertainty remains. Briefly the experiments, which will be published later, have shown that sulphide is formed by hydrolysis of thiocyanate under conditions which do not permit oxidation. Sulphide is also oxidized by washed cell suspensions. With all these substrates except thiocyanate there was a decrease in the pH value of the medium during growth, due to the formation of sulphate ion. When thiocyanate was used ammonia was also produced and the pH change did not occur.

The thiocyanate organism, growing with thiocyanate, provides an unusual example of an organism which is able to use one simple inorganic compound as its source of energy, of nitrogen and, indirectly, of carbon. The initial step of thiocyanate metabolism is the hydrolysis of thiocyanate to sulphide, ammonia and carbon dioxide. In respiratory experiments theoretical amounts of carbon dioxide are not produced, and it appears probable that the organism can obtain its carbon requirements by hydrolysis of thiocyanate and subsequent absorption of carbon dioxide. This will be discussed more fully in a later paper. It will be clear from these findings that it was not possible to demonstrate directly the requirement by the organism of carbon dioxide when growing on thiocyanate, since the removal of carbon dioxide by KOH, supposing it could be sufficiently rapid or complete, would be accompanied by the removal of the sulphide which was also essential. It was, however, possible to demonstrate the requirement of carbon dioxide for growth on thiosulphate. This was accomplished by inoculating and incubating two similar flasks of mineral salts medium containing phosphate buffer, ammonium sulphate and sodium thiosulphate. One flask was incubated in air, the other in a desiccator in the bottom of which was placed a 10 % solution of KOH. Fluted filter-papers were used to increase the area of KOH exposed. When the cultures, thus deprived of carbon dioxide, failed to grow over a period of ten or more times that required by the controls, they were removed and incubated in the air as a test that the inoculum had been satisfactory. Growth then occurred.

An attempt was made to test nitrate and nitrite as nitrogen sources, but it was not possible to decrease the ammonia concentration sufficiently to prevent growth in the controls. The experiment did show that nitrite was inhibitory at low concentrations (100 p.p.m.) and that some reduction of nitrate to nitrite occurred.

A requirement for phosphate was shown using media, contained in carefully washed glassware and prepared from triple distilled water, with analytical grade reagents in minimal amounts. After two subcultures no growth occurred in the control medium to which no addition was made, or in those to which 0·2 μg. P/ml. had been added; the addition of 0·4 μg. P/ml. allowed growth. High phosphate concentrations were inhibitory; growth did not occur above 1266 μg./ml. and the growth rate was reduced at 950 μg./ml. For general purposes 0·01 M-phosphate was satisfactory. It was advisable to increase the buffer concentration when using a substrate from which acid was produced, e.g. thiosulphate. Iron and magnesium were shown to be necessary by experiments similar to those described for phosphate. The effects of magnesium deficiency were more marked than those of iron deficiency.

The optimum temperature for growth is *c.* 30°, with an upper limit of 37° and growth is found over the range 15–37°. The pH optimum for growth on thiocyanate, thiosulphate and sulphur was between pH 7·0 and 7·2, although there was little variation in the rate of growth over the range pH 6·8–7·6.

The optimum concentration of thiocyanate is 0·0025 M if judged by the length of time which elapsed before visible growth appeared. At 0·005 M, growth appeared later but the final growth was greater. Growth also occurred at 0·01 M but inhibition was complete at 0·02 M.

DISCUSSION

It now seems possible to place the organism in the genus *Thiobacillus* and to call it *T. thiocyanoxidans*, replacing the non-committal name, *Bacterium thiocyanoxidans* used hitherto. The genus *Thiobacillus* is characterized by the ability to grow with carbon dioxide as the sole source of carbon and inorganic compounds of sulphur as the source of energy. Our organism fulfils these requirements. It will grow in a medium containing only phosphate, trace metals and thiocyanate. A nitrogen source is added in the form of ammonium sulphate to obtain growth on thiosulphate or sulphur; with the latter two compounds carbon dioxide must be present in the gas phase. The organism also shows sensitivity to organic compounds.

A specimen of this organism has been lodged with the National Collection of Industrial Bacteria.

We are indebted to Dr G. Eaves for the electron micrographs and to Mr J. B. Kirkham for photography. One of us (J.B.Y.) held a grant from the Gas Research Council when this work was undertaken.

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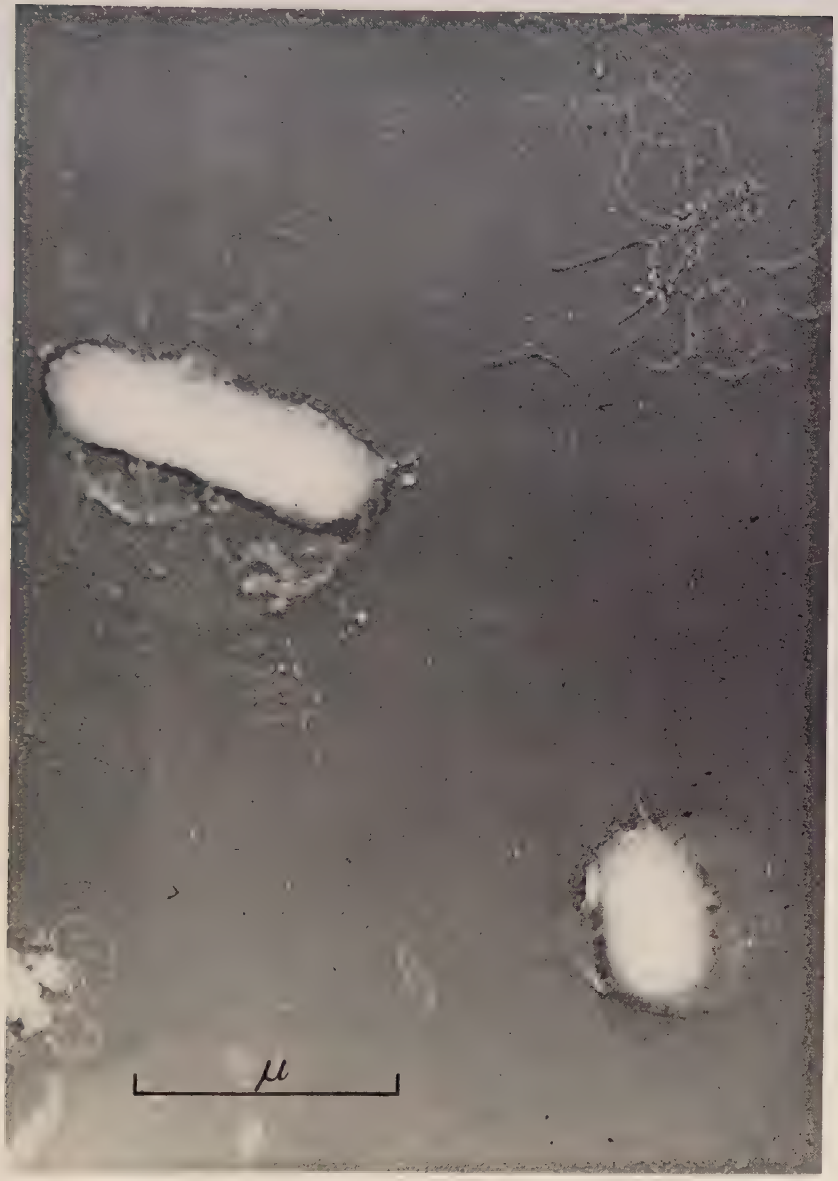
EXPLANATION OF PLATE

Electron micrograph of a gold-shadowed preparation of *Thiobacillus thiocyanoxidans*, showing the polar flagellum.

(*Received* 24 *August* 1953)



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The Folic Acid and Serine Nutrition of *Leuconostoc mesenteroides* P60 (*Streptococcus equinus* P60)

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SUMMARY: *Leuconostoc mesenteroides* P60 (*Streptococcus equinus* P60) requires serine for growth under normal conditions. On a medium containing relatively high concentrations of glycine it can dispense with serine provided that (*a*) pyridoxal is present, and (*b*) either Leucovorin (folinic acid) is added or the atmosphere is enriched with CO_2. Increasing concentrations of CO_2 or Leucovorin decrease the concentration of glycine required to support growth without added serine. Pyridoxal is not necessary for optimal growth with added serine, but the required concentration of the latter is twice as great.

Higher concentrations of *p*-aminobenzoic acid or Leucovorin are required for growth on glycine than on serine. Leucovorin replaces *p*-aminobenzoic acid for growth on serine, and both this factor and CO_2 for growth on glycine. With the basal medium used (containing purines and all other amino-acids) thymidine supports growth in the absence of *p*-aminobenzoic acid only when serine is added.

Growth is not inhibited by sulphonamide when Leucovorin is present; with *p*-aminobenzoic acid there is the usual competition. Pteroylglutamic acid is inactive with this organism.

A valid assay for serine is possible, even with high concentrations of glycine present, when precautions are taken to destroy either Leucovorin or pyridoxal in the samples, and to prevent the atmosphere becoming enriched with CO_2.

In connexion with work on the synthesis of serine by cell suspensions of *Streptococcus faecalis* R (Lascelles & Woods, to be published) it was necessary to devise an assay for small quantities of serine in the presence of relatively high concentrations of glycine and of members of the folic acid group of growth factors. Attention was given to microbiological assay since chemical methods proved insufficiently sensitive or otherwise unsatisfactory with the type of material to be analysed. Amongst micro-organisms reported to require serine for growth, *Leuconostoc mesenteroides* P 60 has been frequently used or suggested for assay of serine (Dunn, 1949; Steele, Sauberlich, Reynolds & Baumann, 1949; Barton-Wright, 1952). The nutritional requirement of this organism for serine was therefore investigated in detail, particularly in relation to the effect of various folic acid derivatives and of varying amounts of glycine. Some of these results have already been briefly reported (Lascelles & Woods, 1950; Lascelles, Cross & Woods, 1951).

It was found that, under certain conditions of growth, *Ln. mesenteroides* P 60 could synthesize serine for itself. Nevertheless, it was possible to devise a valid assay by taking steps to ensure that such conditions did not exist. The growth experiments upon which these conclusions are based are reported in detail since they also yielded considerable information concerning the final stage of serine synthesis by this organism. The literature bearing on this matter will be considered in the Discussion in relation to the present results.

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* Beit Memorial Fellow at the time of this work.

MATERIALS AND METHODS

The organism. Leuconostoc mesenteroides P 60 (American Type Culture Collection, no. 7881) was maintained in stab culture on medium B1 of Nimmo-Smith, Lascelles & Woods (1948). Stock cultures were subcultivated fortnightly and incubated 24 hr. at 37°; they were stored at 2°. It has recently been reported (McCleskey, 1952) that strain P60 of this organism is improperly placed in the genus *Leuconostoc* and is closely related to *Streptococcus equinus*. The original name is retained here (even if incorrect) to avoid confusion in reference to earlier work.

Media. The chemically-defined basal medium (medium L) was a modification of that used by Sauberlich & Baumann (1948) for the assay of *Ln. citrovorum* factor with that organism. In particular, the concentration of DL-alanine was increased since this overcame the inhibition of growth which occurred in the absence of pyridoxal when the serine concentration exceeded 2×10^{-4}M.

Medium L contained (per litre final volume): DL-alanine, 450 mg.; L-arginine hydrochloride, 200 mg.; DL-aspartic acid, 200 mg.; L-asparagine, 400 mg.; DL-cysteine hydrochloride, 130 mg.; DL-glutamic acid, 600 mg.; L-histidine hydrochloride, 60 mg.; DL-isoleucine, 240 mg.; DL-leucine, 240 mg.; DL-lysine hydrochloride, 400 mg.; DL-methionine, 100 mg.; DL-phenylalanine, 100 mg.; DL-proline, 200 mg.; DL-threonine, 200 mg.; DL-tryptophan, 40 mg.; DL-tyrosine, 200 mg.; DL-valine, 240 mg.; glucose, 20 g.; sodium acetate trihydrate, 20 g.; NH_4Cl, 3 g.; KH_2PO_4, 0·6 g.; $Na_2HPO_4.12H_2O$, 0·6 g.; $MgSO_4.7H_2O$, 0·4 g.; $MnSO_4.4H_2O$, 20 mg.; $FeSO_4.7H_2O$, 20 mg.; adenine, guanine, xanthine and uracil, each 10 mg.; riboflavin, 0·5 mg.; nicotinic acid, 1 mg.; calcium pantothenate, 0·6 mg.; thiamine hydrochloride, 0·6 mg.; biotin, 1 μg. Before the addition of the other constituents the amino-acids were dissolved in 40 ml. N-NaOH, the solution diluted to 200 ml. and brought to pH 6–7 with 10N-HCl. The complete medium (quantities for 1 l.) was adjusted to pH 6·8, boiled gently for 3 min., filtered and diluted to 500 ml. (double strength medium L).

In tests designed to show the effect of *p*-aminobenzoic acid and related substances it was necessary to treat the amino acids with charcoal. After dissolving in N-NaOH as above the solution was brought to pH 3 with 10N-HCl; 2 g. charcoal (Norit) was added, the mixture held at 100° for 45 min. and filtered.

Medium L_S was used for the assay of serine. It was prepared from medium L by adding (final concn.) 5×10^{-4}M-glycine; 10^{-6}M-*p*-aminobenzoic acid, 10^{-7}M-pteroylglutamic acid and, when desired, 2×10^{-7}M-pyridoxal.

Procedure. The double strength medium (L or L_S) was distributed in 1 ml. quantities in hard glass tubes ($5 \times \frac{5}{8}$ in.). After adding test materials and water to a total volume of 1·9 ml., the tubes were autoclaved 7 min. at 10 lb./sq.in. Leucovorin (synthetic *Ln. citrovorum* factor) and pyridoxal were added when required as sterile solutions after the autoclaving.

The inoculum was derived from a fresh stab culture in medium B1 which was subcultured into tryptose-glucose broth (medium B2, Nimmo-Smith *et al.* 1948) and incubated 24 hr. at 37°. The cells were centrifuged out, suspended in the original culture volume of water and diluted 1/50; each tube of test medium was inoculated with 0·1 ml. (about 4×10^6 cells/ml. final medium). For experiments on the effect of *p*-aminobenzoic acid and derivatives it was necessary first to wash the cells three times with the culture volume of 0·85 % (w/v) NaCl.

Incubation was at 37° for 42–46 hr. For atmospheres other than air the adapted 'Kilner' fruit-preserving jars referred to by Nimmo-Smith *et al.* (1948) were used.

The extent of growth is recorded as the galvanometer reading given by an EEL photoelectric colorimeter (Evans Electroselenium Co.) using 0·25 in. tubes and a neutral density filter; the uninoculated medium was used to obtain the zero setting. A culture containing 0·29 mg. dry weight cells/ml. gave a reading of 10; there was a linear relationship between reading and dry weight of cells up to a reading of 35.

Special chemicals. 'Leucovorin' (Ca salt pentahydrate, 99 % purity) was a gift from Dr H. P. Broquist (Lederle Laboratories); a stock solution (10^{-3}M) was prepared in sterile water; it was not further sterilized. 'Aldehyde-free' pteroylglutamic acid was kindly supplied by Dr T. H. Jukes of the same Laboratories; a 10^{-3}M stock solution was prepared by dissolving the solid in 10^{-2}M-$NaHCO_3$. Pyridoxal hydrochloride was a gift from Dr K. Folkers (Merck Inc.); the stock solution in water (2×10^{-3}M) was sterilized by Seitz-filtration. All three solutions were stored at −20° in the dark. Thymidine was a gift from Dr E. E. Snell and L-serine from Dr A. Neuberger.

RESULTS

Effect of carbon dioxide on the ability of glycine to replace serine

When cultures were incubated in air, N_2 or H_2, serine was essential for growth even with high concentrations of glycine present. In atmospheres containing 5 % (v/v) CO_2, however, serine was not required provided there was a sufficiently high concentration of glycine (Table 1).

The concentration of glycine required to give a given growth response in the absence of serine was a function of the partial pressure of CO_2 (Fig. 1*a*); as the latter was increased the necessary amount of glycine decreased. Maximum growth in the presence of 5 % CO_2 was reached with 0·01 M-glycine.

With an optimal concentration of serine present added CO_2 was not needed (Table 1). When, however, the serine concentration was suboptimal, the presence of CO_2 increased growth markedly (Fig. 1*b*); under these conditions it is probable, therefore, that the available serine is supplemented by synthesis from glycine.

Replacement of CO_2 *by dicarboxylic acids.* Citrate, oxaloacetate and α-ketoglutarate, but only at concentrations of 0·01 M and above, partially replaced the need for added CO_2 for growth with glycine (Table 1, Exp. 2). Other carboxylic acids, including formate, were without effect. Certain dicarboxylic acids have been shown to replace CO_2 for the growth of *Escherichia coli* (Lwoff & Monod, 1947; Ajl & Werkman, 1948).

Table 1. *Effect of carbon dioxide and certain carboxylic acids on growth in the presence of serine or glycine*

Medium L was supplemented with *p*-aminobenzoic acid (10^{-6} M), pteroylglutamic acid (10^{-7} M), pyridoxal (2×10^{-7} M) and the other substances shown. The carboxylic acids were added as the sodium salts (pH 6·8); oxaloacetate and α-ketoglutarate were sterilized by filtration. When citrate was added the medium was further supplemented with (μg./ml.) $MgSO_4.7H_2O$, 400; $MnSO_4.4H_2O$, 80; $FeSO_4.(NH_4)_2SO_4.6H_2O$, 30. Incubation 43 hr. in the atmosphere shown.

			Growth (EEL reading) with added	
Exp.	Atmosphere (5 % (v/v) CO_2 when present)	Carboxylic acid ($2{\cdot}5 \times 10^{-2}$ M)	Glycine (10^{-2} M)	Serine* (10^{-3} M)
1	Air	—	0	31
	Air/CO_2	—	26	30
	H_2	—	2	29
	H_2/CO_2	—	28	30
2	N_2	—	0	28
	N_2/CO_2	—	28	30
	N_2	Formate	4	—
	N_2	Malate	5	—
	N_2	Fumarate	5	—
	N_2	Succinate	3	—
	N_2	Citrate	13†	—
	N_2	Oxaloacetate	15†	—
	N_2	α-Ketoglutarate	9†	—

* Glycine (5×10^{-4} M) was also present (see text).
† No growth occurred when glycine was omitted.

Relative activity of serine and glycine. In the presence of added CO_2 the concentration of glycine required for optimal growth was about 50 times that of serine (cf. Fig. 1*a*, *b*). With serine present, and whether or not incubation was in an atmosphere enriched with CO_2, glycine was still essential, though the molar concentration was diminished to 0·02–0·05 of that required with serine absent (Fig. 2). This requirement for glycine when serine is present in excess shows that the organism cannot convert serine to glycine (if it does so at all) at a rate sufficient to support growth; it can, however, carry out the converse reaction when the atmosphere is enriched with CO_2. Certain animal tissues are able actively to convert serine to glycine (Shemin, 1946).

Requirement for pyridoxal. When serine was present in optimal concentration pyridoxal increased growth only slightly; it was essential for growth when glycine plus CO_2 replaced serine (Table 2, Exp. 1). This was still true at the limiting effective concentrations of both glycine and CO_2 (Table 2, Exp. 2). Pyridoxal is therefore required, directly or indirectly, for the conversion of glycine to serine.

When serine was present, but in suboptimal concentration, pyridoxal markedly increased growth: about twice as much serine was required for

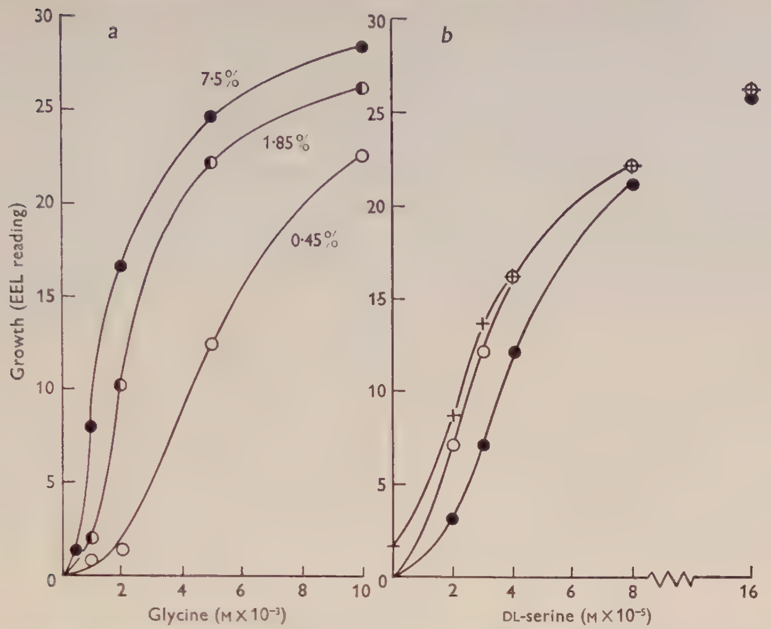

Fig. 1*a*. Effect of concentration of glycine and of carbon dioxide on growth without serine. Medium L was supplemented with 10^{-6} M-*p*-aminobenzoic acid, 10^{-6} M-pteroylglutamic acid and 2×10^{-7} M-pyridoxal. Incubation 44 hr. in atmospheres of N_2 containing the % (v/v) CO_2 shown.

Fig. 1*b*. Effect of CO_2 and Leucovorin on growth with various concentrations of serine. Medium L contained supplements as in Fig. 1*a* and 5×10^{-4} M-glycine. Incubation 44 hr. in N_2 (●—●), 5% CO_2 in N_2 (○—○), N_2 with medium containing 10^{-7} M-Leucovorin (+—+).

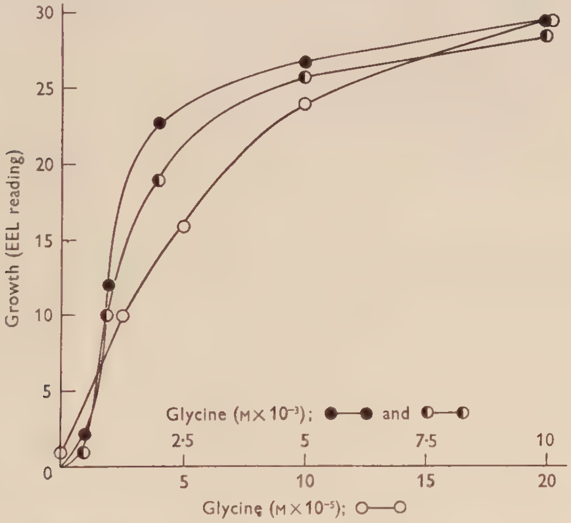

Fig. 2. Relative requirement of glycine growth in the presence and absence of serine. Medium L was supplemented as in Fig. 1*a*. Incubation 44 hr. in H_2 (containing CO_2 where indicated). Serine absent: 5% CO_2 (●—●) or 10^{-7} M-Leucovorin (◐—◐). Serine present (○—○).

a given growth response in the absence of pyridoxal as in its presence (Fig. 3). Similar results were obtained with both DL- and L-serine, though the latter was twice as active as the racemic form whether or not pyridoxal was present.

Table 2. *Effect of pyridoxal on growth in the presence of glycine and serine*

Medium L was supplemented with *p*-aminobenzoic acid (10^{-6} M), pteroylglutamic acid (10^{-7} M) and the other substances shown. Incubation 44 hr. in atmosphere shown.

				Growth (EEL reading) with pyridoxal (2×10^{-7} M)	
Exp.	Atmosphere	Glycine (M)	Serine (M)	Absent	Present
1	5 % CO_2 in H_2	5×10^{-4}	—	0	2
		10^{-2}	—	0	26
		5×10^{-4}	10^{-3}	24	29
2	0·06 % CO_2 in N_2	2×10^{-2}	—	1	11
	20 % CO_2 in N_2	10^{-3}	—	0	17

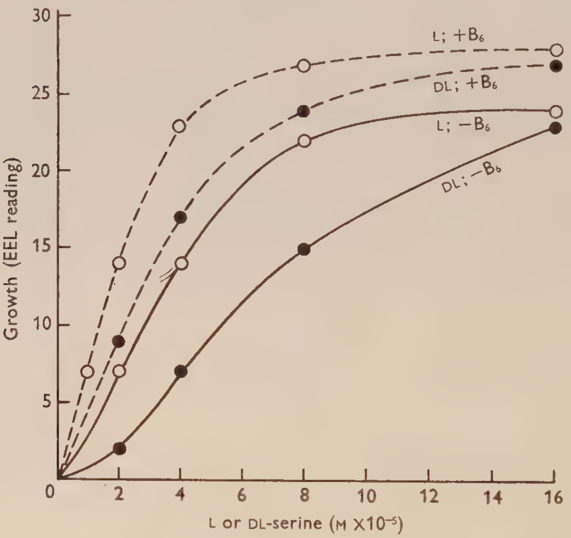

Fig. 3. Response of *Ln. mesenteroides* P60 to L- and DL-serine in the presence and absence of pyridoxal (B6). Medium L was supplemented with 10^{-6} M-*p*-aminobenzoic acid, 10^{-7} M-pteroylglutamic acid and 5×10^{-4} M-glycine. Pyridoxal (2×10^{-7} M) present (----) or absent (——); L-serine (○), DL-serine (●). Incubation 44 hr. in air.

It appears that this organism cannot use the D-isomer. The increased stimulation of growth by pyridoxal at limiting serine concentrations again suggests that under these conditions the supply of serine is supplemented by synthesis from glycine.

Requirement for members of the folic acid group

Pennington (1946) first reported *p*-aminobenzoic acid to be essential for the growth of *Ln. mesenteroides* P60; other strains of this organism which do not respond to *p*-aminobenzoic acid have been found to need pteroylglutamic acid (Whiteside-Carlson & Carlson, 1949). The requirement of the present strain for members of the folic acid group was tested on medium L, based on charcoal-treated amino acids (p. 268). Best growth on this medium was about 70 % of that on the medium containing untreated amino acids.

p-*Aminobenzoic acid.* This substance was essential for growth either on a medium containing serine or on one in which serine was replaced (as above) by high concentrations of glycine and a CO_2-enriched atmosphere. Under the latter conditions, however, the quantitative requirement for *p*-aminobenzoic acid was increased to about 5 times that when serine was supplied (Fig. 4*a*). An atmosphere containing added CO_2 did not influence the concentration of *p*-aminobenzoic acid required for growth with serine present. The requirement for *p*-aminobenzoic acid was maintained over the whole range of effective concentrations of glycine and CO_2; thus in the limiting conditions of 2×10^{-2} M-glycine with 0·065 % CO_2 and 10^{-3} M-glycine with 20 % CO_2 (each giving only half-optimal growth) the need was still absolute. The increased need for *p*-aminobenzoic acid for growth in the absence of serine provides evidence that the factor (or some derivative) is concerned in the conversion of glycine to serine as well as in other reactions essential for growth.

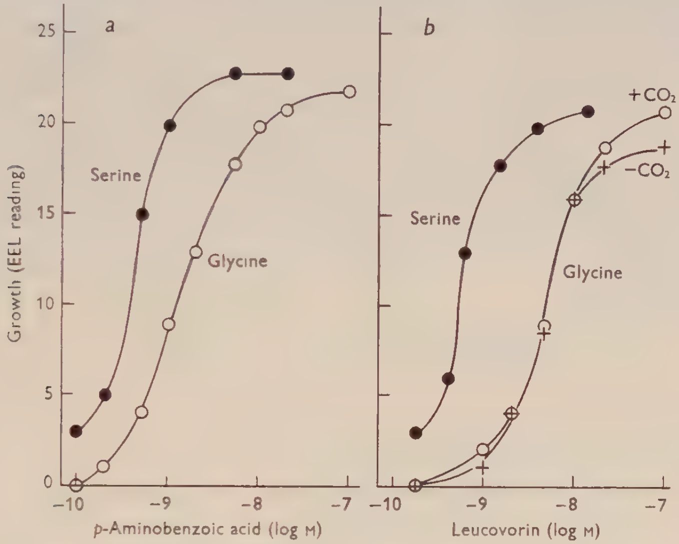

Fig. 4*a* and *b*. Response of *Ln. mesenteroides* P60 to *p*-aminobenzoic acid (*a*) and Leucovorin (*b*) for growth in serine or high concentrations of glycine. Medium L was supplemented with 2×10^{-7} M-pyridoxal and 10^{-3} M-DL-serine plus 5×10^{-4} M-glycine (●—●) or 10^{-2} M-glycine (○—○, +—+). Incubation 44 hr. in 5 % CO_2 in N_2 (○—○, ●—●) or N_2 only (+—+).

Other members of the group. Neither pteroylglutamic acid nor its N^{10}-formyl derivative supported growth at concentrations up to a thousand times that effective with *p*-aminobenzoic acid; this was true both for growth with serine and with glycine plus CO_2 (Table 3). Pteroic acid and rhizopterin

(N^{10}-formylpteroic acid) were also inactive. It is probable, therefore, either that these substances do not permeate the cell or that the organism cannot convert them (as it presumably does *p*-aminobenzoic acid) to the form in which folic acid ultimately has function in metabolism. Experiments with the N^5-formyltetrahydro derivative of pteroylglutamic acid (Leucovorin), which did replace *p*-aminobenzoic acid for growth, are discussed later.

Table 3. *Replacement of* p-*aminobenzoic acid by members of the folic acid group and by thymidine*

Medium L was based on charcoal-treated amino acids and was supplemented with 2×10^{-7} M-pyridoxal and the substances shown. Incubation 44 hr. in 5% (v/v) CO_2 in H_2.

Addition	Concn. (M)	Growth (EEL reading) in presence of Glycine (10^{-2} M)	Serine* (10^{-3} M)
None	10^{-7}	0	2
p-Aminobenzoic acid		18	20
Pteroylglutamic acid		0	3
N^{10}-Formylpteroylglutamic acid		0	3
Leucovorin		18	18
Thymine	5×10^{-6}	0	4
Thymidine		0	20

* Glycine (5×10^{-4} M) was also added.

Replacement of p-*aminobenzoic acid by thymidine.* Thymine or thymidine has frequently been found to be a component of mixtures which will support the growth of organisms requiring members of the folic acid group in the absence of these factors. Shive, Eakin, Harding, Ravel & Sutherland (1948) found the inhibition of growth of a strain of *Ln. mesenteroides* by a methylpteroylglutamic acid to be overcome by thymidine, but not thymine, as well as by pteroylglutamic acid.

With the present strain thymidine supported growth in the absence of *p*-aminobenzoic acid when the medium contained serine, but not when the latter was replaced by glycine plus CO_2 (Table 3); thymine was inactive, which suggests that the organism cannot convert it to the deoxyriboside. When the medium (which already contains purine and an amino acid mixture) is supplemented with serine and thymidine it apparently contains all metabolites in the synthesis of which *p*-aminobenzoic acid is concerned. When, however, the supply of serine is dependent on formation from glycine, *p*-aminobenzoic acid is still required; this confirms the specific function of the growth factor in the conversion of glycine to serine. With *Strep. faecalis* and a similar type of medium, Broquist & Snell (see Snell, 1951) found the growth requirement for folic acid to be abolished when thymine and serine were added; without thymine, folic acid was again required and the need was increased tenfold when serine was also omitted.

Experiments with Leucovorin

There is now very strong evidence that the naturally occurring factor required for the growth of *Ln. citrovorum* (ATCC 8081)* is (−)L-5-formyl-5,6,7,8-tetrahydropteroylglutamic acid. The synthetic material (Leucovorin, folinic acid-SF) is a mixture of the (+)L- and (−)L-isomers of this compound and has half the activity of the crystalline natural factor (Roth, Hultquist, Fahrenbach, Cosulich, Broquist, Brockman, Smith, Parker, Stokstad & Jukes, 1952; Cosulich, Smith & Broquist, 1952; Sauberlich, 1952; Pohland, Flynn, Jones & Shive, 1951; Bond, Bardos, Sibley & Shive, 1949; Keresztesy & Silverman, 1951). Leucovorin also replaces pteroylglutamate for the growth of *Strep. faecalis* R and *Lactobacillus casei*, but its ability to promote growth of organisms responding to *p*-aminobenzoic acid has not been reported.

Table 4. *Growth with high concentration of glycine: replacement of* CO_2 *and* p-*aminobenzoic acid by Leucovorin*

Medium L (based on charcoal-treated amino acids) was supplemented with 10^{-2} M-glycine and the substances shown. Incubation: 44 hr. in atmosphere stated.

'Acid-treated' Leucovorin was prepared by bringing an aqueous solution to pH 2 with N-HCl, autoclaving at 10 lb. pressure/sq.in. for 7 min. and neutralizing (pH 7) with sterile N-NaOH; it was added to the sterile basal medium.

		Additions				
Exp.	Atmosphere	*p*-Amino-benzoate (10^{-7} M)	Leucovorin (10^{-7} M)	Leucovorin 'acid-treated' (10^{-7} M)	Pyridoxal (2×10^{-7} M)	Growth (EEL reading)
	N_2	—	—	—	+	0
		+	—	—	+	1
		—	+	—	+	18
		—	+	—	—	0
	5 % CO_2 in N_2	—	—	—	+	0
		+	—	—	+	20
		+	—	—	—	0
		—	+	—	+	21
		—	+	—	—	0
2	N_2	+	—	—	+	1
		—	+	—	+	18
		—	—	+	+	2
		+	—	+	+	3
	5 % CO_2 in N_2	—	—	—	+	0
		+	—	—	+	18
		—	+	—	+	19
		—	—	+	+	16

Replacement of p-*aminobenzoic acid and of* CO_2. Leucovorin had a dual effect with *Ln. mesenteroides* P60. First, it replaced *p*-aminobenzoic acid both for growth with added serine and for growth with glycine plus CO_2 (serine absent). Secondly, it abolished the need for added CO_2 under the latter conditions; with optimal concentrations of Leucovorin (10^{-8} M and above) growth with 0·01 M-glycine in an atmosphere of N_2 was equal to that found with *p*-aminobenzoic acid or Leucovorin in an atmosphere enriched with CO_2 (Fig. 4*b*, Table 4).

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* It has recently been reported (Felton & Niven, 1953) that this organism is incorrectly identified as *Ln. citrovorum*; it is a typical strain of *Pediococcus cerevisiae*.

In a series of experiments Leucovorin was 2–5 times less active on a molar basis than *p*-aminobenzoic acid in supporting growth either with serine or high concentrations of glycine (cf. Fig. 4*a* with 4*b*); thus the concentrations required for half-maximal growth were (Leucovorin first): $5{\cdot}4 \times 10^{-10}$ M and $3{\cdot}6 \times 10^{-10}$ M with serine, and $6{\cdot}4 \times 10^{-9}$ M and $1{\cdot}4 \times 10^{-9}$ M with glycine. These figures are taken from the experiments of Fig. 4 with a gas phase containing CO_2 in each case. The presence of CO_2 had no significant effect on the quantitative requirement for either growth factor when serine was present, or on that for Leucovorin for growth on glycine (Fig. 4*b*); it was as usual required for growth on glycine with *p*-aminobenzoic acid.

A difference of a factor of two between the intrinsic activities of Leucovorin and *p*-aminobenzoic acid is possible since the former contains only 50 % of the natural isomeride.

The growth factor activity of Leucovorin for *Ln. citrovorum* is lost by autoclaving it at pH 2. After such treatment (Table 4, Exp. 2) it no longer replaced CO_2 for growth on the glycine medium (serine absent), but it retained the property of replacing *p*-aminobenzoic acid under the conditions where the latter was active (presence of serine or of glycine plus CO_2). Acid treatment of Leucovorin is known to result in a mixture of products (Cosulich, Roth, Smith, Hultquist & Parker, 1951), but further interpretation of the present results is not possible in view of the complexity of the chemical changes occurring during such treatment.

Effect of glycine concentration on Leucovorin requirement. The concentration of glycine required for growth (serine absent) with Leucovorin was almost identical with that required when growth was promoted by *p*-aminobenzoic acid plus CO_2 (Fig. 2); it was again about 50 times greater than that when serine was present. The concentration of glycine needed bore a reciprocal relation to the concentration of Leucovorin added (Fig. 5); a reduced quantity of glycine could be compensated (within limits) by increased Leucovorin. This effect is essentially similar to that observed earlier with CO_2 (Fig. 1*a*).

Requirement for pyridoxal for growth on glycine. The replacement of *p*-aminobenzoic acid by Leucovorin did not modify the absolute requirement for pyridoxal for growth (serine absent) with high concentrations of glycine (Table 4).

Effect of Leucovorin on inhibition of growth by sulphathiazole. The fact that Leucovorin replaces *p*-aminobenzoic acid for the growth of *Ln. mesenteroides* P 60 suggests that the latter substance plays its part in cell metabolism through intermediate conversion to Leucovorin or a closely similar folic acid derivative. Since, however, the concentration of Leucovorin required was slightly higher than *p*-aminobenzoic acid, other interpretations were possible, and confirmation was sought by comparing the effects of the two factors on inhibition of growth by sulphathiazole on media containing either serine or high concentrations of glycine. There was, as anticipated, a strictly competitive relationship between *p*-aminobenzoic acid and the sulphonamide over a 1000-fold range of concentration of the latter (Fig. 6). With Leucovorin present (at just optimal concentration for growth), the organism was almost completely insensitive to sulphathiazole, even at the highest concentration (Fig. 6). This would be expected if Leucovorin is the product of utilization of *p*-aminobenzoic acid whose formation is prevented by sulphonamide.

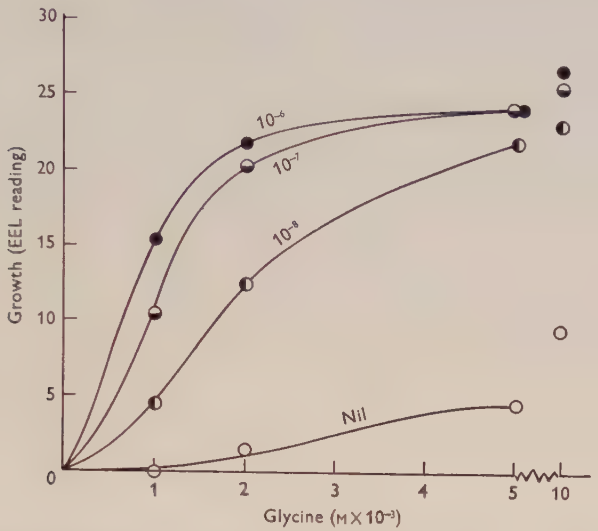

Fig. 5. Effect of concentration of Leucovorin on the requirement for glycine (serine absent). Medium L plus 2×10^{-7} M-pyridoxal, 10^{-6} M-*p*-aminobenzoic acid and the concentration (M) of Leucovorin shown on each curve. Incubation, 45 hr. in N_2.

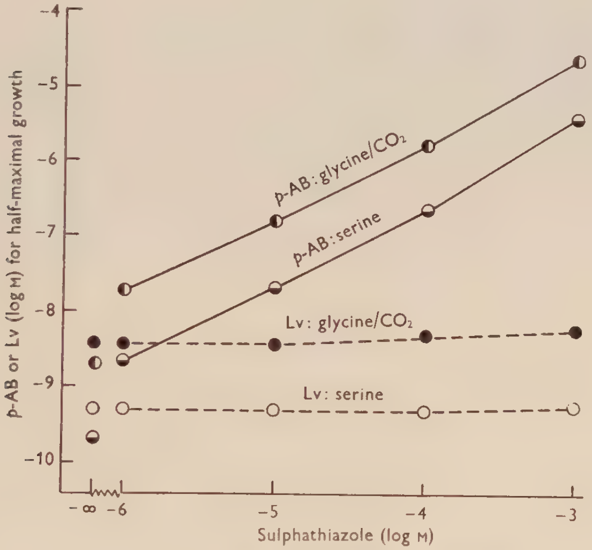

Fig. 6. Ability of *p*-aminobenzoic acid and Leucovorin to overcome inhibition of growth by sulphathiazole. Medium L (charcoal-treated amino-acids) plus 2×10^{-7} M-pyridoxal and supplemented with *either* (*a*) 10^{-3} M-serine + 5×10^{-4} M-glycine and incubated in N_2 (○, ◒), *or* (*b*) 10^{-2} M-glycine and incubated in 5 % CO_2 in N_2 (●, ◐). Leucovorin (Lv), – – – –; *p*-aminobenzoic acid (*p*-AB), ——.

The basal medium used in this work contained an amino acid mixture (except serine and glycine) and purines; the deduction that *p*-aminobenzoic acid functions through conversion to a Leucovorin-like substance can therefore only strictly be applied to its role in the synthesis of thymidine (results on serine medium) and in the conversion of glycine to serine (results on the high glycine medium).

Serine content of organisms grown in serine-free medium

It has been assumed in previous sections that when the organism grew in a serine-free medium containing high concentrations of glycine (with added CO_2 or Leucovorin) it was able to do so by synthesizing the required serine from glycine. This is a reasonable assumption in view of the likelihood that bacterial protein must contain serine in all circumstances. However, the matter was tested experimentally in the case of cells grown in glycine plus CO_2 by comparing their serine content with that of normal cells.

Medium L (no serine) was supplemented with 0·01 M-glycine and the required amounts of *p*-aminobenzoic acid and pyridoxal for growth under these conditions. After inoculation the culture was incubated in 5 % CO_2 in N_2. To avoid any possible carry-over of serine, the inoculum was derived from the twelfth successive subculture of the organism on the same medium. The cells were harvested at full growth by centrifuging, and washed once in phosphate buffer. A sample of the final cell suspension was hydrolysed by heating in 6 N-HCl in a sealed tube for 17 hr. at 105°. The hydrolysate was neutralized and assayed for serine by the microbiological assay described below. Control cells were derived from culture on a medium based on acid-hydrolysed casein (which contains serine) and were treated in the same way. The serine contents were (% serine of dry wt.): 0·9 for cells grown in glycine plus CO_2; 1·0 for the control cells. These are minimum values since some serine is known to be lost during acid hydrolysis. The interest here however is in the comparative rather than absolute contents.

The presence of serine in the hydrolysate was confirmed chromatographically after conversion to the dinitrophenyl derivative (Campbell & Work, 1952). There is no doubt therefore that cells grown in the presence of glycine and CO_2 do, in fact, synthesize serine.

The assay of serine

It is clear from Fig. 3 that medium L is a suitable one for the assay of serine; thus with excess pyridoxal present the dose-response relationship is linear for the range 0·5 to 4×10^{-5} M-L-serine. The preceding work has shown, however, that the presence of pyridoxal, glycine or Leucovorin-like substances in the material under test, or of CO_2 in the atmosphere, might seriously affect the validity of the assay.

Effect of pyridoxal. A valid assay would be obtained either with pyridoxal totally absent or present in excess. If the basal medium contains no pyridoxal (which is not essential for growth), any contribution of pyridoxal from the material under test would lead to erroneously high figures for serine (Fig. 3).

Excess pyridoxal (see Methods) should therefore be added for routine assays; it must be omitted, however, for special tests described later.

Effect of glycine. Even at the low concentration at which it is itself required for optimal growth, glycine increases growth with suboptimal serine, but only when the atmosphere contains CO_2 or when Leucovorin is present (Fig. 1*b*). If further glycine is added with the material assayed, this effect becomes more serious since the required concentration of CO_2 (Fig. 1*a*) or Leucovorin (Fig. 5) is then reduced, and growth may occur even in the absence of serine. For a valid assay it is therefore essential to remove Leucovorin or like substances from the test material and to make sure that the atmosphere of incubation does not become enriched with CO_2.

Control of CO_2. *Leuconostoc mesenteroides* P60 produces only traces of CO_2 during growth on medium L_s and gives a homolactic fermentation of glucose (McCleskey, 1952; and present work). A sufficiently low concentration of CO_2 in the gas phase is attained by incubation of the tubes in a closed vessel filled with N_2 or H_2; the vessel should not however contain too many tubes. Incubation in air has sometimes been found to lead to erratic results. Although the normal CO_2 content of fresh air (0·03 %) would not interfere, the slightly higher content to be expected in a closed incubator containing other cultures may approach the critical level of 0·06 % effective with 10^{-2} M-glycine (Table 2). Citrate, oxaloacetate and α-ketoglutarate are not normally likely to be present in material to be assayed at the high concentrations at which they replace CO_2. It is clear that a basal medium containing citrate cannot be used if the test material is likely to contain relatively large amounts of glycine.

Removal of Ln. citrovorum *factor.* Treatment of Leucovorin with dilute acid destroys its activity both for *Ln. citrovorum* (Broquist, Stokstad & Jukes, 1950) and in the present sense (Table 4). All material for assay should therefore be treated as in Exp. 2 of Table 4. It has been found, however, that complete removal is not certain when Leucovorin is added to bacterial extracts at concentrations exceeding 10^{-6} M. With each new type of material it is necessary to check (by specific assay with *Ln. citrovorum*) that this factor has been completely destroyed.

Assay in the absence of pyridoxal. When serine is absent, pyridoxal is essential for growth at any concentration of glycine even when CO_2 or Leucovorin is also present (Tables 2 and 4); serine, however, supports growth in the absence of pyridoxal. An assay in the absence of pyridoxal, even though less sensitive (Fig. 3), should always be used as a further check on the validity of results obtained as above. Pyridoxal is omitted from medium L_s and the samples for assay treated to destroy members of the vitamin B_6 group. Steele *et al.* (1949), who had found these substances to stimulate growth of *Ln. mesenteroides* in the absence of serine, destroyed them by submitting materials for assay to ultraviolet irradiation. Pyridoxal can also be inactivated by autoclaving with tryptophan (Snell, 1945), and this method was used here. The experiment of Table 5 shows that the treatment is efficient and also results in no loss of serine. A solution of serine autoclaved with pyridoxal and tryptophan gave the same growth response as an untreated solution; the removal of pyridoxal is shown by the fact that growth is halved when pyridoxal is absent from the assay medium (Table 5, cf. Fig. 3).

Table 5. *Destruction of pyridoxal by autoclaving with tryptophan*

A solution containing DL-serine (10^{-3}M), DL-tryptophan (10^{-2}M) and pyridoxal (10^{-6}M) was autoclaved at a pressure of 15 lb./sq.in. for 30 min. The treated solution was compared with DL-serine of equivalent molarity for its ability to support growth on medium L_8 with and without pyridoxal. Incubation: 44 hr. in air.

Supplement (ml.)*		Growth (EEL reading) with pyridoxal (2×10^{-7}M)	
Treated solution	DL-serine (10^{-3}M)	Absent	Present
0	0	0	0
0·1	0	8	16
0·2	0	17	23
0·4	0	24	26
0	0·1	9	16
0	0·2	16	24
0	0·4	25	28

* Final volume of each culture was 2 ml.

DISCUSSION

Glycine as precursor of serine. The fact that glycine (albeit at rather high concentrations) is able to replace serine under certain conditions for the growth of *Ln. mesenteroides* P60, and that cells grown in this way have a normal content of serine, provides strong evidence that this organism is able to convert glycine to serine. The possibility that glycine may be a normal intermediate in serine synthesis by micro-organisms has previously been suggested by observations that certain induced mutants of Neurospora and *E. coli* will grow if given either serine or glycine (Tatum, 1949; Roepke, Libby & Small, 1944; Wright, 1951). Further support for a direct origin of serine from glycine comes from a finding that, when *Torulopsis utilis* is grown on a medium containing [*carboxy*-^{14}C] glycine as sole source of carbon, the bulk of the isotope is found in the serine fraction of the hydrolysed cell-protein (Ehrensvärd, Sperber, Saluste, Reio & Stjernholm, 1947). In animal tissues Sakami (1948) has shown by isotope technique that C-2 of glycine is incorporated into C-2 of serine; C-3 of the latter may be derived from several methyl-group donors including formate and glycine itself (Sakami, 1948, 1949; Siegel & Lafaye, 1950).

Function of folic acid in the conversion of glycine to serine. The increased quantitative requirement for *p*-aminobenzoic acid (or Leucovorin) in growth under conditions where serine must be derived from glycine clearly indicates a specific function of folic acid at this step in serine synthesis. The ability of certain mixtures of amino acids and nucleic acid derivatives to replace the growth factor function of *p*-aminobenzoic acid and pteroylglutamic acid (and the anti-sulphonamide function of the former) had earlier suggested that folic acid was required at some stage in serine formation (see reviews by Shive, 1951; Woods, 1952); serine was specifically implicated by Lampen, Jones & Roepke (1949) and Winkler & de Haan (1948) in the case of *p*-aminobenzoic acid and by Broquist & Snell (see Snell, 1951) in the case of pteroylglutamic acid. Wold & Sirny (1953) briefly reported that Leucovorin will replace serine for the growth of *Ln. mesenteroides* P60. Their basal medium contained glycine and citrate, and, as would be expected from the work reported here, supported some growth without added serine; the need for the latter was made absolute in their tests by increasing the concentration of L-alanine present. It was also mentioned that a relatively high glycine content increased growth in the serine-free medium.

A function of folic acid in the conversion of glycine to serine has received further indirect support from experiments with growing cultures of *Lactobacillus bifidus* (Nepple, Wright & Skeggs, 1951) and *Tetrahymena pyriformis* (*geleii*) (Kidder & Dewey, 1953). With the former organism folic acid would not replace serine unless glycine were present, while with the latter glycine replaced serine only when relatively high concentrations of folic acid were added. Evidence that the reaction promoted here by folic acid is one in which a one-carbon residue is transferred comes from Plaut, Betheil & Lardy (1950) who found that the incorporation of ^{14}C from formate into C-3 of serine was markedly less in folic acid-deficient rats than in normal animals.

Metabolic relationship of p-*aminobenzoic acid and Leucovorin.* It has been seen that Leucovorin can replace *p*-aminobenzoic acid for the growth of *Ln. mesenteroides* P60; slightly higher molar concentrations of the former are required than would be expected simply on a basis of a 50% content of an inactive isomeride and a molecular equivalence with *p*-aminobenzoic acid. Nevertheless, the behaviour of the two growth factors in the presence of sulphonamide (Fig. 6) is in accord with that expected if Leucovorin (or a similar substance) is the sole product of the metabolism of *p*-aminobenzoic acid by the cell. Pteroylglutamic acid, however, is without activity either in replacing *p*-aminobenzoic acid or overcoming sulphonamide inhibition. Unless it be supposed that pteroylglutamate (though not the closely related Leucovorin) is unable to permeate the cell, a reasonable explanation of these results would be that this organism is able to convert either *p*-aminobenzoic acid or Leucovorin, but not pteroylglutamic acid, to the ultimate coenzyme form of folic acid; i.e. that pteroylglutamate is not a direct intermediate in the synthesis of the coenzyme.

Leuconostoc mesenteroides P60 is unique among organisms so far tested which require *p*-aminobenzoic acid, in that Leucovorin is active when pteroylglutamate is not. There is considerable variation among such organisms in the ability of pteroylglutamic acid to replace *p*-aminobenzoic acid and to render the organism insensitive to sulphonamides (Woods, 1950); but in all other cases the two forms of folic acid have both been either inactive or active for a given test organism (unpublished observations, see Woods, 1952). It would not be justifiable therefore to deduce from the present work either that Leucovorin is a normal intermediate in the synthesis of a coenzyme form of folic acid, or that it is itself that coenzyme. These matters are discussed more fully elsewhere (Woods, 1952, 1953).

Requirement for vitamin B_6 *in serine synthesis.* Pyridoxal was found to be essential for growth whenever the organism was obliged to synthesize serine from glycine (Tables 2 and 4), though not when exogenous serine was supplied. It is probable therefore that this factor, presumably as pyridoxal phosphate, is also required for the glycine → serine transformation. Deodhar & Sakami (1953) briefly reported evidence that incorporation of [^{14}C] formate into serine by chick tissues is dependent on vitamin B_6. Earlier work had also suggested that vitamin B_6 was needed at some stage in serine synthesis. *Strep. faecalis* R was found to require serine for growth (in an otherwise complete amino acid medium) only when pyridoxin was lacking, while growth of both *Lactobacillus arabinosus* and *Ln. mesenteroides* in the absence of this amino acid was stimulated by members of the vitamin B_6 group (Lyman, Moseley, Wood, Butler & Hale, 1947; Steele *et al.* 1949).

Although *Ln. mesenteroides* P60 grew fully without addition of pyridoxal when serine was present, the concentration of this amino acid then required was double that needed when pyridoxal was also added (Fig. 3). No explanation has been found for this; since the same result was obtained with both DL- and L-serine the possibility that pyridoxal permitted the organism to use the D-isomer appears to be excluded. Vitamin B_6 derivatives are known to have such action with other amino acids, and pyridoxal phosphate has been shown to be the coenzyme of an alanine racemase enzyme system (Lyman & Kuiken, 1948; Wood & Gunsalus, 1951).

Effect of carbon dioxide. A more detailed analysis of the need for CO_2 or Leucovorin for growth with glycine (serine absent) will be published elsewhere and has been reported briefly (Cross, 1953). The inverse quantitative relationship between the required concentrations of glycine and CO_2 (Fig. 1*a*) led to the suggestion that CO_2 might serve, with growing cultures, as a source of the necessary one-carbon residue for condensation with glycine (Lascelles *et al.* 1951); later experiments with $^{14}CO_2$ have shown however that this is not the case. Leucovorin replaces CO_2 in this effect and there is again an inverse quantitative relationship with glycine. The quantity of serine found in the cells after growth in such conditions showed that it was impossible that the formyl group of Leucovorin itself was the ultimate source of the C-3 of serine; at least 200 molecules of serine were formed for each molecule of Leucovorin present at the time of inoculation.

We are grateful to Sir Rudolph Peters, F.R.S., for his interest in this work. One of us (M.J.C.) was indebted to the Ministry of Education for a grant for further education and training.

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(*Received* 31 *August* 1953)

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A Study of the Mechanism of Inhibition of Bacteriophage Multiplication by Chymotrypsin

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SUMMARY: When 0·01 % chymotrypsin is added to mixtures of rhizobium bacteriophage and bacteria in liquid cultures, the multiplication of phage is prevented and the phage gradually becomes inactive. The rate and extent to which phage and bacteria combine are unaffected by the chymotrypsin whose effect seems directed against an early stage in the interaction between the two. This stage persists, on average, for less than 1 min. from the moment of combination. A phage particle combined with a bacterium becomes inactive and the bacterium is thus protected against lysis and remains able to multiply. Chymotrypsin does not interfere with combination between phage and bacteria killed by ultraviolet radiation; the combination leads to loss of phage activity irrespective of the presence of chymotrypsin. The multiplication of the phage is unaffected by previous incubation of either the phage or of the host bacteria separately with 0·01 % chymotrypsin.

Chymotrypsin has no effect on phage-host interaction in an agar medium.

Ribonuclease, which apparently has no direct effect on a rhizobium bacteriophage, can nevertheless inhibit its multiplication in liquid bacterial cultures, probably by affecting host bacteria (Kleczkowski & Kleczkowski, 1952). Chymotrypsin has now been found to be a much more efficient inhibitor of multiplication of the phage, although it does not affect directly either the phage or the bacteria in their abilities to interact with each other normally. The inhibitory effect of chymotrypsin, whose study is described in this paper, is directed against an early stage in the phage-host interaction.

MATERIAL AND METHODS

A phage which attacks a strain of clover nodule bacteria (*Rhizobium trifolii*, strain 317) was used. The media and the method of 'poured plates' for obtaining plaques were described previously (Kleczkowska, 1945*a*).

Active phage was assayed by suitably diluting the fluids to be tested in 24 hr. liquid bacterial cultures; 1 ml. samples of this were mixed with 9 ml. of 0·7 % agar medium, melted and cooled to 42°, and poured into a Petri dish of about 10 cm. diameter. Plaques were counted after 48 hr. incubation at 25°. When fluids contained bacteria and phage, these assays gave, of course, totals of 'active centres', i.e. of free phage and of infected bacteria.

Phage concentrations given below are mean numbers of plaques formed by 1 ml. samples of the diluted fluids, multiplied by the reciprocal of the dilutions.

Conclusions drawn from the results obtained in this work are unaffected by 'plating efficiency', i.e. by the magnitude of the ratio of estimated to actual phage concentrations, assuming this ratio to have been constant. It was probably near 1·0 (Kleczkowski & Kleczkowski, 1951).

Phage stock cultures were lysed liquid bacterial cultures passed through a Chamberland L3 filter. They usually contained about 10^8 estimated phage particles/ml.

Liquid bacterial cultures were made by inoculating a loopful from an agar culture into 9 ml. of sterile liquid medium which was then incubated at 25°. After 24 hr. incubation the cultures contained *c.* 5×10^6 bacteria/ml. and after 48 hr. *c.* 10^7/ml.

Crystallized chymotrypsin was prepared from fresh beef pancreas as described by Northrop, Kunitz & Herriot (1948). Chymotrypsinogen was recrystallized five times, then turned into chymotrypsin and recrystallized twice. Solutions of the crystals were dialysed for 48 hr. in cellophan sacks against frequently changed distilled water and then dried at 37° in a flat dish placed in a desiccator. Weighed quantities of the dry material were dissolved in water immediately before use. In all experiments the pH of the various mixtures was between 7·5 and 7·7.

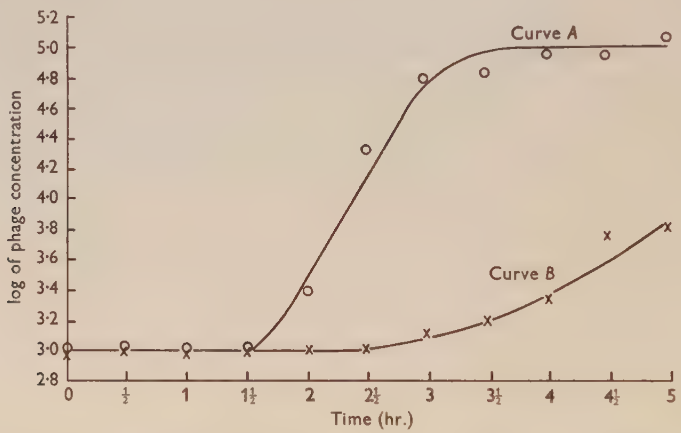

Fig. 1. The course of phage multiplication in liquid culture. Curve A: phage multiplication when there was no disturbance, i.e. when each sample for assay was taken from a different tube. Curve B: phage multiplication when a culture was disturbed by stirring during the taking of samples at 30 min. intervals.

RESULTS

Combination and multiplication of phage

The designs of all main experiments in this work were based on three features of phage-host interaction which must be remembered in the interpretation of the results. First, the relatively slow rate of combination of the phage with the host bacteria (Table 4). Secondly, the 90 min. duration of the latent period (at 25°) followed by 90 min. period of liberation of phage from infected cells, each cell producing on average about 100 phage particles (curve *A*, Fig. 1). Thirdly, the decrease in the rate of phage multiplication caused by frequent disturbances of cultures. The effect of these disturbances is seen from comparison of curves *A* and *B*, Fig. 1. Curve *A* shows phage multiplication when there was no disturbance, when samples for each assay were taken from different tubes. Curve *B* was obtained when all samples were taken from the same tube which was thus disturbed every 30 min. Whether the stirring inseparable from taking representative samples affects combination between phage and bacteria, or disturbs development of phage within bacterial cells, is unknown, but, as Fig. 1 shows, the effect is considerable and must be allowed for in designing experiments.

Effect of chymotrypsin (*CTR*)

Table 1 shows that the presence of CTR in solidified agar medium did not prevent plaques forming or appreciably affect their number until the concentration of CTR in the fluid to be mixed with the agar medium and plated reached 0·1 % (i.e. 0·01 % in the agar medium), when the number was decreased by about 25 %. Thus, plaque counts can be used to assay concentration of active phage in the presence of CTR, provided the plated bacterial culture contains less than 0·1 % CTR and that the plating is done immediately after dilution in the culture of the fluid to be tested. When the plated culture contains more than 0·001 % CTR, delay in plating allows a proportion of phage to combine with the bacteria and become inactivated, thus decreasing the number of plaques.

The inability of 0·01 % CTR to prevent plaque formation (test no. 2, Table 1) contrasts sharply with its ability to stop phage multiplication in liquid bacterial cultures. Similarly, its ability to inactivate phage in liquid bacterial cultures (test no. 1, Table 2) contrasts with its inability to do so in the absence of bacteria (test no. 6, Table 2). It is not clear why CTR cannot act similarly in agar medium. Its adsorption by agar, its too slow diffusion through the agar, or the formation of a protective coating of agar on the bacterial surface are possible reasons.

Table 1. *Plaque formation in the presence of chymotrypsin*

Test no.	Composition of mixtures: 24 hr. bacterial culture (ml.)	Chymotrypsin (ml.): 1·0 %	Chymotrypsin (ml.): 0·1 %	Chymotrypsin (ml.): 0·01 %	H_2O (ml.)	Phage at a dilution of of 10^{-5} (ml.)	Nos. of plaques on four plates
1	4	0·5	—	—	—	0·5	320
2	4	—	0·5	—	—	0·5	415
3	4	—	—	0·5	—	0·5	420
4	4	—	—	—	0·5	0·5	440

9 ml. of 0·7 % agar medium melted and cooled to 42° was mixed with 1 ml. samples of the above mixture and poured into Petri dishes.

The rate of bacterial multiplication in liquid cultures was not affected by CTR at concentrations up to 0·1 %. The only observed effects of CTR on bacteria were that in 0·1 %, but not in 0·01 % CTR, the bacteria tended to form loose aggregates composed of a few cells, and that at both concentrations of CTR the motile cells that normally occur were not produced.

To see whether CTR alters bacteria and makes them unable to interact normally with the phage, 24 hr. bacterial cultures were incubated for a further 24 hr. with 0·01 % CTR, the cells were then spun down, suspended in fresh medium and phage added to the suspension. Phage multiplied in the suspension at the same rate as did phage added to a comparable bacterial suspension which had not been exposed to CTR.

Table 2. *Effect of chymotrypsin on phage multiplication in liquid cultures*

Test no.	Composition of mixtures: 24 hr. bacterial culture (ml.)	Medium (ml.)	Chymotrypsin (ml.): 0·1 %	0·04 %	0·01 %	0·0025 %	H_2O (ml.)	Concentration of active phage aft: 0 hr.	3 hr.	24 hr.	48 hr.
1	4	—	0·5	—	—	—	—	650	50	3	0
2	4	—	—	0·5	—	—	—	670	250	6200	40 × 10
3	4	—	—	—	0·5	—	—	660	6500	50×10^8	—
4	4	—	—	—	—	0·5	—	700	8×10^4	45×10^8	35 × 10
5	4	—	—	—	—	—	0·5	700	8×10^4	45×10^8	35 × 10
6	—	4	0·5	—	—	—	—		—	—	690
7	—	4	—	—	—	—	0·5		—	—	680

0·5 ml. of phage diluted 10^{-5} was added to each mixture which was then incubated at 25°.

Table 3. *Combination between phage and bacteria in the presence of chymotrypsin*

Composition of mixtures: 24 hr. bacterial culture (ml.)	H_2O (ml.)	0·1 % CTR (ml.)	Phage at 10^{-4} (ml.)	After ½ hr. incubation at 25°		Test no.	Mean numbers of plaques per plate
3·2	0·4	—	0·4	2 ml. not centrifuged		1	112
				2 ml. centrifuged*	supernatant	2	42
					sediment†	3	64
3·2	—	0·4	0·4	2 ml. not centrifuged		4	35
				2 ml. centrifuged*	supernatant	5	40
					sediment†	6	8

* For 15 min. at 10,000 r.p.m.

† Suspended in 2 ml. of medium.

All the resulting fluids were plated at a dilution of 1/100. The numbers of plaques are averages of four plates.

As chymotrypsin does not seem to affect separately either phage or host bacteria in their abilities to interact with each other normally, it presumably affects directly the course of their interaction. The possibility that it interferes with combination between phage and bacteria was excluded as it did not alter the amounts of phage remaining in the supernatant fluids when phage + bacteria mixtures were centrifuged to remove the bacteria (compare tests nos. 2 and 5, Table 3). Furthermore, the concentration of active phage in the CTR-containing mixtures remained unaffected by the removal of the bacteria (compare tests nos. 4 and 5, Table 3). The phage that had combined with bacteria had been rendered inactive by CTR. This effect of CTR could not be reversed by removing phage-infected bacteria from CTR-containing medium and placing them in fresh medium (compare tests nos. 3 and 6, Table 3). Most or all of the phage that was combined with bacteria in the presence of CTR remained inactive. The few plaques obtained in test no. 6 could have resulted from contamination of the bacterial sediment with uncombined phage or from activity of a small proportion of combined phage.

To see how long CTR takes to inactivate phage combined with bacteria, phage + bacteria mixtures, with and without CTR, were diluted at intervals to 1/100, which brought the concentration of CTR well below its limiting effective level and also decreased very considerably the rate of further combination between phage and bacteria (Table 4). Phage was assayed after 4 hr. incubation.

In test no. 9 the bacterial culture and phage had both been diluted 1/1000 before they were mixed (the mixture C). The amount of phage formed at the end of 4 hr. incubation is assumed to be the amount that combined with bacteria, in time to multiply and be released by the end of the incubation, multiplied by 100, plus the amount that did not combine. The phage increased 3·1 times, so that if ρ is the proportion that combined, $100\rho+(1-\rho)=3{\cdot}1$, therefore ρ is about 0·02. It is assumed, therefore, that after the mixtures A and B of Table 4 had been diluted 1/1000, the proportion of phage, still remaining free, that combined with bacteria subsequently and multiplied was not greater than 0·02. This is small in relation to proportions (P) combined before the dilutions were made and is therefore neglected in computing these proportions for tests nos. 6–8, where there was no CTR.

The proportions of phage still active at the time of dilution, despite being combined in the presence of 0·01 % CTR (i.e. before the mixture A was diluted), were computed by making two assumptions. First, that the proportion (P) combined was unaffected by CTR. Secondly, that the proportion (ρ) of phage, still remaining free when the dilution was made, which combined subsequently and multiplied in time to be released by the end of the incubation, was about 0·02 in tests nos. 2 and 3, and between 0 and 0·02 in test no. 4 in which the dilution was longer delayed, so that the proportion of remaining free phage which could combine and multiply after the dilution was considerably smaller than 0·02.

The proportion (x) of phage, combined before the dilution, that still multiplied, is then computed from the equation

$$100xP+100\rho(1-P)+(1-\rho)(1-P)=r,$$

where r is the ratio of phage concentration at the end of the incubation to 130, which was the initial concentration.

Table 4 shows that most of the phage that combined with bacteria in the presence of 0·01 % CTR did not multiply, although the concentration of CTR was later much below its limiting effective level. Only a small proportion of combined phage multiplied when the dilution was made within 10 min. of mixing, and the proportion decreased further as the time was increased.

To see whether later stages of phage-host interaction can be affected, CTR was added to a mixture of phage and bacteria at various intervals after the two were mixed. Table 5 shows that in contrast to the decrease in concentration of active phage that occurred during 4 hr. incubation in mixtures in which CTR was added to a bacterial culture before or together with phage, the concentration of active phage increased when CTR was added after adding phage, the increase being the greater the longer the interval of time between adding phage and CTR. (Even when the interval was decreased to the shortest possible, which was about 1 min., the concentration of active phage increased slightly during 4 hr. incubation.) However, after the initial increases the concentrations of active phage dropped subsequently, which was apparent when a second assay was made after 24 hr. incubation.

Table 4. *Effect of interrupting the action of chymotrypsin by dilution*

Composition of mixtures: A: 3·2 ml. of 48 hr. bacterial culture + 0·4 ml. of 0·1 % CTR + 0·4 ml. phage diluted 10^{-3}. B: 3·2 ml. of 48 hr. bacterial culture + 0·4 ml. H_2O + 0·4 ml. phage diluted 10^{-3}. C: 3·6 ml. of 48 hr. bacterial culture diluted 10^{-3} + 0·4 ml. phage diluted 10^{-6}.

Test no.		Concentration of active phage after 0 hr.	Concentration of active phage after 4 hr.	Proportion of phage combined before dilution (P)	Assumed proportion of remaining phage combined after dilution (ρ)	Proportion of phage, combined before dilution, that multiplied (x)
1	A undiluted	11×10^4	$0·6 \times 10^4$	—	—	—
2	A dil. 10^{-3} after 10 min.	(110)*	500	(0·2)†	0·02	0·07
3	A dil. 10^{-3} after 30 min.		350	(0·5)†	0·02	0·025
4	A dil. 10^{-3} after 90 min.		100	(0·9)†	0–0·02	0·005–0·008
5	B undiluted	13×10^4	13×10^6	—	—	—
6	B dil. 10^{-3} after 10 min.	(130)*	2900	0·2	—	1·0
7	B dil. 10^{-3} after 30 min.		6500	0·5	—	
8	B dil. 10^{-3} after 90 min.		11500	0·9	—	
9	C undiluted	(130)*	400	—	—	—

All the dilutions were made in the medium.

The fluids were kept at room temperature (20°) until the last dilutions of A and B were made in tests nos. 4 and 8. Then all the fluids were incubated at 25°.

* The values of tests no. 1 and 5, respectively.

† The values assumed equal to those of tests nos. 6, 7 and 8, respectively.

Evidently it is only during a minute or so, after combination between a phage particle and a bacterial cell has taken place, that they can be affected by CTR. Then a change occurs, possibly the entry of phage material into the cell or a process preliminary to it, after which CTR cannot affect the further processes leading to normal phage multiplication within the cell and the release of phage into the surrounding medium. As the time between adding phage and CTR increased, the proportion of phage that had combined with bacteria and passed the vulnerable stage also increased and, consequently, the proportion of phage that could multiply increased. However, the newly formed phage, released into the medium, combined with bacteria in the presence of CTR and became inactive. Hence the subsequent decrease following the initial increase in the concentration of active phage. Even the initial increase was underestimated by the assay, because by the time it was made a proportion of newly formed phage had combined with bacteria and become inactive.

Table 5. *Effect of varying time between adding phage and chymotrypsin to a culture of host bacteria*

The mixtures contained 4 ml. of a 24 hr. bacterial culture, 0·5 ml. of phage at a dilution of 10^{-5} and 0·5 ml. of 0·1 % CTR (the control had 0·5 ml. H_2O instead of CTR), and were incubated at 25°.

	Concentration of active phage after		
CTR added:	0 hr.	4 hr.	24 hr.
1 hr. before phage	750	90	20
Together with phage	700	110	20
5 min. after phage	1,100	2,000	20
15 min. after phage		7,000	230
45 min. after phage		20,000	430
Control (no CTR)		97,000	95×10^7

It is concluded that the CTR acts upon a freshly formed phage cell combination with the result that the phage particle combined with a bacterial cell remains inactive. There is at present no evidence to show whether the state of inactivity is the result of a structural alteration in the phage particle or of a reaction affecting only its junction with the cell, so that the particle cannot continue its normal interaction with the cell. Bacterial cells, on the other hand, which combined with phage in the presence of 0·01 % CTR remained able to multiply (Table 6).

Table 6. *Ability of bacterial cells combined with phage in the presence of CTR to multiply and form colonies*

The mixtures were incubated for 1 hr. at 25° and then diluted 10^{-5}. One ml. samples of these dilutions were then mixed with 9 ml. of 0·7 % agar medium, melted and cooled to 42°, and poured into Petri dishes. Bacterial colonies were counted after 5 days incubation at 25°.

Composition of mixtures: 2 ml. of 24 hr. bacterial culture +				
0·1 % CTR (ml.)	H_2O (ml.)	Phage (undil.) (ml.)	Medium (ml.)	Mean nos. of colonies per plate
0·5	—	2	—	115
—	0·5	2	—	6
0·5	—	—	2	125
—	0·5	—	2	120

When the concentration of CTR in a liquid bacterial culture was 0·005 %, phage multiplication was not inhibited completely and permanently, but was delayed for 2–3 days (test no. 2, Table 2). Then the culture lysed and phage reached about the same concentration as in the absence of CTR.

Proteolytic activity of CTR in the lysed culture was assayed by a method based on rendering casein unprecipitable by trichloroacetic acid, and was found to be unaltered during incubation. The delayed phage multiplication did not, therefore, occur because of a decrease in the concentration of active CTR. The fact that the whole culture was lysed shows that it was not a bacterial mutant that interacted with the phage, for a mutant would have formed only a proportion of the culture. The phage that did multiply in the presence of CTR was not a mutant immune to the inhibitory effect of CTR as it was tested and found as susceptible as phage formed in the absence of CTR.

It is concluded that phage cell interaction can proceed in the presence of 0·005 % CTR, though its progress is then greatly slowed. The limiting concentration below which CTR has no effect, seems to be *c.* 0·001 % (test nos. 3 and 4, Table 2).

It is not certain what happens when the concentration of CTR in a liquid medium is within the range where it does not stop phage multiplication completely but only delays it. The delay may occur because some stage of the interaction between all phage particles and bacteria is slowed or because some phage particles are inactivated, whereas a proportion interact at the normal rate.

The effect of CTR on phage-host interaction seems to be connected with its proteolytic activity, as neither chymotrypsinogen nor CTR whose proteolytic activity has been destroyed by heat, or by exposure to ultraviolet radiation, had any effect on phage-host interaction.

Effects of killing bacteria by heat or ultraviolet radiation

To check the conclusion that CTR inactivates the phage only when it is interacting with bacteria, experiments were made with bacteria killed by exposure to heat or to ultraviolet radiation.

Heating 48 hr. bacterial cultures, containing *c.* 5×10^6 bact./ml., for 5 min. at 100° destroyed the ability of bacteria to combine specifically with phage, although heat-killed bacteria, when used as more concentrated suspensions, can adsorb unspecifically small proportions of phage added to them (Kleczkowska, 1945*b*). Phage added to this heated culture remained free, its concentration unaltered even after incubation for several hours at 25° and the addition of CTR had no effect.

Ultraviolet-killed bacteria may retain their ability to combine specifically with their phages (Garen & Puck, 1951) and the ultraviolet-killed bacteria used in this work did retain it, although the rate of combination was somewhat decreased as a result of irradiation. A 48 hr. bacterial culture was irradiated so that the proportion of bacterial cells still able to form colonies was $1{\cdot}5 \times 10^{-5}$ (see Table 7). When phage was added to this culture and assayed after 4 hr. incubation at 25°, the concentration of active phage was only about 14 % of that added and this concentration remained unaltered when the ultraviolet-killed bacteria were removed by centrifugation and the supernatant fluid was tested. Evidently phage that combined with ultraviolet-killed bacteria became inactive; it probably remained attached to them and so was not available to live bacteria. There is, therefore, a similarity between the result of combination of phage with ultraviolet-killed bacteria and with live bacteria in the presence of 0·01 % CTR.

Table 7. *Combination between phage and bacteria killed by ultraviolet radiation*

	Composition of mixtures				Concentration of active phage		
	48 hr. bacterial culture					After 4 hr. incubation at 25°	
Mixture no.	Non-irradiated (ml.)	Irradiated (ml.)	0·1 % CTR (ml.)	H_2O (ml.)	Initially	Non-centrifuged culture	Supernatant fluid
1	4	—	0·5	—	730	50	—
2	4	—	—	0·5		75,000	—
3	—	4	0·5	—		95	100
4	—	4	—	0·5		100	95

0·5 ml. of phage stock culture diluted 10^{-5} added to each mixture, which was then incubated for 4 hr. at 25°. Samples of mixtures nos. 3 and 4 were then centrifuged for 15 min. at 10,000 r.p.m. Phage was assayed in non-centrifuged cultures and in supernatant fluids of centrifuged samples.

The presence of 0·01 % CTR in a mixture of phage and ultraviolet-killed bacteria had no effect on the phage. The rate of the latter's combination with the bacteria was unaffected by CTR, and combined phage became inactive irrespective of whether CTR was present or not.

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(*Received* 31 *August* 1953)

Brieger, E. M., Cosslett, V. E. & Glauert, A. M. (1954). *J. gen. Microbiol.* **10**, 294–303.

Reproductive Changes in Avian Tubercle Bacilli studied with the Electron Microscope

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SUMMARY: The various stages of the reproductive cycle in freshly transplanted cultures of avian tubercle bacilli were recorded by a series of successive electron micrographs. An early stage of cytoplasmic differentiation is described in which discrete cellular units form inside the elongating filaments. Their significance and relation to the vacuolization seen at later stages is discussed. Evidence is produced to show that the dense bodies of mycobacteria are not nuclei. Free granules do not appear to play any part in the reproductive cycle in the normal type of development. In mycelial variants lysis is often seen to initiate new growth which starts from submicroscopical elements and micro-rods.

The electron microscope has already produced valuable information about the internal structure of tubercle bacilli in various stages of development. However, the results obtained still lack coherence and have given rise to conflicting interpretations of the cytological details observed. In particular there has been controversy concerning the nature of the dense spherical bodies which seem to be a characteristic of the species of *Mycobacterium* although they are also present in related species (Mudd, Brodie, Winterscheid, Hartman, Beutner & McLean, 1951). They were first described by Lembke & Ruska (1940) and have been variously identified as nuclei (Knaysi, Hillier & Fabricant, 1950), nucleoids or karyoids (Ruska, Bringmann, Neckel & Schuster, 1952), mitochondria (Mudd, Winterscheid, DeLamater & Henderson, 1951), metachromatic granules (Werner, 1951) and skeletal structures for the support of nucleic acids, phosphates, lipoids, waxes, etc. (Lembke, 1947). Again there is considerable difference of opinion as to the nature of the intracellular organization seen at various stages of development (Brieger, Crowe & Cosslett, 1947; Brieger & Cosslett, 1949; Knaysi *et al.* 1950; Ruska *et al.* 1952).

The correlation of morphological and intracellular changes with stages in the reproductive process requires an almost continuous series of electron micrographs covering every phase of the development. The present paper describes results obtained by paying particular attention to the early stages of development of various avian strains grown on electron microscope grids placed on a variety of media.

MATERIALS AND METHODS

Six avian strains were investigated with the electron microscope. Of these two (Bone and Dobson) were highly virulent, two (AF and Lister) were avirulent and one (Fn) showed declining virulence. All except the Lister strain were freshly recovered from tuberculous birds. The present paper deals mainly with observations on the Fn strain which was compared with the virulent Sheard strain used by American workers.

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Technique

In early work we used a stripping technique (Brieger *et al.* 1947) in which the bacilli were removed from a solid medium by covering it with a thin collodion membrane which was then floated off on water. Although giving a satisfactory picture of individual organisms this method suffers from the defect that an unknown proportion of the growth remains on the medium. Also, the amyl acetate used as a solvent in laying down the collodion film may induce changes in internal structure. Later a method was developed of growing bacilli directly on collodion-covered electron microscope grids (Brieger & Cosslett, 1949) and this has recently been modified for use with liquid media.

Stainless steel electron microscope grids were used, as it was suspected that copper grids would have an inhibitory effect on the growth of the bacilli. The grids were sterilized and covered with a thin formvar membrane in the usual way; formvar was chosen instead of collodion because of its greater strength. Sterile filtered water was used for the preparation of the films to eliminate the contaminating organisms that are invariably present in distilled water.

A thin layer of cotton-wool was placed at the bottom of a small Petri dish (5 cm. diameter, 2 cm. deep) and covered with a disk of filter-paper. After sterilization the cotton-wool was thoroughly soaked with the fluid medium and up to 20 grids placed on the filter-paper with the formvar films uppermost. Each grid was then inoculated with a drop of bacillary suspension in distilled water by means of a fine platinum loop 1 mm. in diameter. If the drop did not stay on the surface of a grid, it was assumed that the holes in the formvar film were too large and the grid was discarded. After the desired time of growth each grid was removed, floated on sterile distilled water to remove any medium adhering to the lower surface, fixed by floating on 5% formalin for 30 min., and washed again to remove any excess fixative. Care was taken throughout the procedure that the inoculated surface of the grid should remain undisturbed. In dealing with pathogenic organisms this method is preferable to that of Hillier, Knaysi & Baker (1948) in which bacilli are grown on a collodion film which is later floated off the medium and mounted on grids.

A preliminary experiment was carried out to show that sufficient nutrient medium diffuses through the formvar film. The growth on the grids was compared with growth directly on the surface of a solid medium, by taking impression prints on clean formvar-covered grids from the growth on a Löwenstein plate. The comparison showed that with Dubos medium the fact that the nutrient fluid had to diffuse through the formvar film did not impair growth to any noticeable extent. A solid medium can be used if a film of saline solution is introduced between the medium and the grid to prevent the formation of an air gap.

Before examination in the electron microscope the grids were viewed with the ×40 dry objective of a phase-contrast microscope so that a comparison of electron and phase-contrast pictures could be made. However, the stainless steel electron microscope grids used for the growth experiments consist of a mesh of woven steel wires and are not flat, so that it was difficult to obtain good phase-contrast photographs. When taking impression prints the flatter copper grids were used, and it was with these that the best phase-contrast photographs were obtained. In future we plan to use flat silver-plated copper grids for the growth experiments.

RESULTS

The reproductive cycle

The general outline of the standard development in micro-cultures of avian tubercle bacilli was known from recent work with the light microscope (Brieger & Fell, 1946), stained preparations (Bretey & Imelik, 1949) and the phase-contrast microscope (Brieger & Glauert, 1952). When a young culture is first transplanted to a fresh medium it consists mainly of short coccoid rods. These elongate into filaments (8–10 μ.) which continue to divide and grow during a phase of filamentous proliferation. After this phase the filaments suddenly break down into masses of short rods which elongate to form the new generation and the cycle is complete. The examination of inoculated grids with the phase-contrast microscope revealed that a similar cycle takes place on the grids, and the observations provide a useful basis for the interpretation of the corresponding electron micrograms. At any one time different parts of the same micro-culture are in different stages of development and, similarly, a variation is also noticeable between one grid square and another. However, the general pattern of growth is uniform throughout, and electron micrographs have been chosen to illustrate every important phase in the development.

The original inoculum. The original suspension was usually taken from a 2- to 3-week old culture growing on Löwenstein's medium, and the inoculum then consisted mainly of well-defined coccoid rods which lay singly or in groups (Pl. 1, fig. 1). The rods varied in length from 0·5 to 1·0 μ. and usually each had two dense raised polar bodies and a uniformly transparent cytoplasm. Evidence for the multiplication of these rods by simple fission was never observed.

Elongation of rods into filaments. During the first 20–24 hr. of incubation the rods of the inoculum began to elongate into filaments of 2–4 μ. in length (Pl. 1, fig. 2*a–d*). In most of the filaments the cytoplasm was seen to be differentiating into a series of separate cellular units each surrounded by a definite limiting membrane. In some filaments the units appeared very dense (Pl. 1, fig. 2*c*) and even caused a swelling of the filament. Sometimes the units formed two groups which retracted away from each other towards the ends of the bacillus (Pl. 1, fig. 3) leaving a comparatively transparent central region. The outer membrane of the whole cell remained unbroken and the filament did not immediately divide. The filaments usually have two fully developed dense bodies in polar positions and in some organisms a number of smaller ones are also seen scattered among the cellular units and apparently associated with them (Pl. 1, fig. 2*d*).

Stage of filamentous proliferation. After the first filaments were formed a rapid proliferation of filaments occured until the grid squares were covered with organisms. The filaments grew and subdivided, frequently remaining attached to each other (Pl. 1, fig. 4). Sometimes they had a characteristic mycelial appearance in phase-contrast photographs (Pl. 2, fig. 5), but when a selected group of organisms from the same grid square was viewed in the electron microscope (Pl. 2, fig. 6) it was seen that the filaments were quite separate, that there was no true branching and that the mycelial appearance was produced because the filaments often remained stuck together. Each daughter filament contained rows of the intracellular units and a number of dense bodies, which in stages of rapid growth were often very small.

The new generation of rods. The final stage in the reproductive cycle led to a massive production of small rods. In the phase-contrast microscope the spaces between the filaments were found to be packed with transparent rods which appeared to have developed from the filaments (Pl. 2, fig. 7). The change occurred very rapidly, and as a result there was no definite evidence in electron micrographs of how the transition from filaments to rods occurred. Sometimes transverse division lines were seen but they were not present in sufficient numbers to indicate that transverse fission was the only means of division. Pl. 2, fig. 8, shows that other processes such as division by retraction or liberation of intracellular units must be considered.

When the cycle was complete, electron micrographs showed masses of rods of fairly uniform size (Pl. 2, fig. 9). Each bacillus usually contained two dense polar bodies, but otherwise the cytoplasm showed little structure and had the uniform appearance seen in the rods of the original inoculum. There are many variations of this cycle depending upon the age of the original culture, the strain and the medium used, but the basic pattern is the same.

Vacuolization

In early observations made with the stripping technique an internal organization was seen in the filaments which consisted of rows of definite units with transparent centres (Pl. 3, fig. 10). At first it was thought that the transparency was due to the solvent action of the amyl acetate used in forming the collodion film, but in later investigations with other techniques similar vacuolized structures were found (Pl. 3, fig. 11*a*, *b*) in the stage of filamentous proliferation. These vacuolized units had a similar location to the intracellular units and in fact all shades of density of the units were observed (Pl. 3, fig. 11*a*). In some cultures the vacuolized units were so numerous that the filaments were packed with transparent bodies each with its limiting membrane (Pl. 3, fig. 12). This vacuolization was seen where conditions were apparently not favourable for normal growth and filaments persist.

Dense bodies

Accompanying the excessive vacuolization of the filaments there was frequently an unusual development of the dense bodies which sometimes grew to giant sizes and filled considerable sections of the filaments (Pl. 3, fig. 13). As a result of even light electron bombardment the bodies were sometimes differentiated into a dark cortex and lighter core (Pl. 3, fig. 14). A similar structure was found in the dense bodies of *Mycobacterium phlei* which sometimes contained two or three lighter areas (Pl. 4, fig. 15).

When growth was very rapid the final rods sometimes had no dense bodies or only very small ones, and a striking arrangement of minute dense bodies was sometimes seen (Pl. 4, fig. 16). Alternatively, the dense bodies outnumbered the filaments which became transparent and the whole field had a spotted appearance (Pl. 4, fig. 17). In such pictures the impression is produced that dense bodies are liberated from the filaments.

Mycelial type of growth

(*a*) When the original inoculum was taken from an old culture which has been growing on Löwenstein's medium for some months it consisted of rods which had merged into clumps (Pl. 4, fig. 18*a*, *b*) and some cells appeared as empty sheaths with no solid content.

The subsequent development of these forms differed from that in young cultures and many amorphous structures were found which contained electron-scattering bodies of various sizes and shapes (Pl. 4, fig. 19). New growth appeared to originate in faint mycelial networks in which pear-shaped structures predominated (Pl. 4, fig. 20). These structures resembled the intracellular units and often had a pair of small dense bodies.

(*b*) In cultures which had wholly reverted to the mycelial type of growth large branched structures were seen which sometimes covered several squares of the grid, and electron micrographs showed that true branching and budding occurred (Pl. 5, fig. 21). The mycelial branches were usually transparent to the electron beam, and there was no evidence of a definite organization similar to that seen in normal filaments. It appears that lateral buds are formed which later grow into rods and then break off. Division also occurred by transverse fission after the appearance of irregularly spaced division lines and the rounding up of the newly formed ends (Pl. 5, fig. 22).

A process of 'lysis' was often seen in mycelial cultures in which the filaments swelled, became less distinct and were surrounded by rods embedded in amorphous and granular material (Pl. 5, fig. 23). In later stages the filaments apparently released large numbers of short bipolar rods, some of which were as small as $0{\cdot}4\,\mu$. (Pl. 5, fig. 24). From phase-contrast studies (Brieger & Glauert, 1952) it is known that this material may be the basis of new growth, but further work is necessary to observe parallel developments in electron micrographs.

Influence of medium

The medium and technique used influenced the type and rate of the resultant growth. Abnormal forms were most common in the later stages of growth on solid media, particularly when there was a formvar film between the culture and the medium. In development on Dubos medium on grids distorted forms were rare. Embryo extract diffuses poorly through the formvar film so that growth is much retarded, and in order to obtain normal growth the grids must be inverted and placed with the inoculated surfaces in contact with the medium. The cytological changes were similar whatever media were used, but the intracellular units were most evident in impression prints taken from growth on Löwenstein's medium.

Comparison of strains. The virulent Sheard strain showed similar developments to the Fn strain, and throughout the experiments there was no obvious difference between virulent and avirulent strains. Consequently, electron micrographs cannot be used as a basis for strain identification.

DISCUSSION

The results presented in this paper provide new evidence of the cytological changes accompanying the reproduction of avian tubercle bacilli. In particular, a striking intracellular organization of the bacilli is described which was not observed by previous workers. It was most obvious during the first 20–30 hr. of incubation when rapid elongation was occurring, and escaped the notice of Ruska *et al.* (1952) who only began their observations after 48 hr. Knaysi *et al.* (1950) show an electron micrograph taken after 21·5 hr. incubation in which the filaments are already highly vacuolized and there is no sign of the intracellular units which in our view precede the vacuolization. Knaysi apparently obtained his material from a fluid medium, in which filaments persist. In order to observe the early phases of growth the material must be removed from a culture which has been growing for no more than 2–3 weeks on a solid medium, when coccoid rods predominate.

The formation of cellular units within the bacillary filaments seems to support the view that mycobacteria are multicellular organisms (Bisset, 1950). However, this organization is only a passing stage coinciding with the multinuclear organization revealed by the HCl+Giemsa method (Brieger & Robinow, 1947). So far as is known there is no parallel development in the bacteria but in the nocardia, which are more closely related to the mycobacteria than is generally believed (Brieger & Glauert, 1952), some sort of analogy can be found in the description of spore formation. 'Spores are formed by the breaking up of the cell plasm into separate portions, usually 3 to 5 in number. Every portion becomes rounded, covered with a membrane, and transforms into a spore; the membrane of the mother cell dissolves and disappears' (Waksman, 1950). Spore formation in the mycobacteria has often been suspected since it was first suggested by Robert Koch, but the idea has been generally discarded. The fact that these units are often associated with a single dot or micro-form of the dense spherical granule seems to support the view that the units represent rods which are being pre-formed inside the filaments (Gróh, 1933). The formation of rods within filaments is a common occurrence in mycelial variants of avian strains (Brieger & Glauert, 1952).

A difficulty arises in relating this intracellular organization to the vacuolization seen at a still later stage. Knaysi *et al.* (1950) stated that these vacuoles 'vary considerably in size, 500 to 3000 A, . . . are unstainable, highly transparent to the electron beam, exert turgor pressure, and accumulate solid content that tends to disappear in old cultures'. From these observations they identified them as 'sap-vacuoles'. The question arises whether the intracellular units and these vacuoles are independent structures or whether they are the same structures in different stages of development. Our observations show that transitional stages exist in which filaments contain intracellular units which have lost electron density and appear as transparent areas (Pl. 3, fig. 11*a*). It thus seems possible that the vacuoles represent intracellular units which have become transparent as a result of chemical changes.

Having identified these structures as 'sap-vacuoles', Knaysi *et al.* (1950) concluded that the dense polar bodies were the nuclei. Ruska *et al.* (1952) also called them nucleoids or karyoids. However, this interpretation conflicts with the view of Robinow & Cosslett (1948) that in bacteria the nuclei are electron transparent. Also Mudd, Winterscheid, DeLamater & Henderson (1951) showed that the dense bodies gave a staining reaction characteristic of mitochondria, and the presence of nuclear structures between the dense bodies has also been demonstrated by means of the Feulgen stain (DeLamater, Hunter & Mudd, 1952). From our observations it would certainly seem more likely that if discrete nuclei exist they should be found between the dense bodies at the site of the newly forming intracellular units.

The function of the dense bodies is still under discussion and their mode of multiplication is also obscure. There can be no doubt that they reproduce rapidly. There is evidence of multiplication by division (Knaysi *et al.* 1950), but one would expect to find many more instances of this division if it were the only means of reproduction (Werner, 1951). A second possibility is that the larger dense bodies fragment into smaller micro-forms, and a third that small dense bodies are synthesized in the cytoplasm (Lembke, 1947), although it is hard to imagine a satisfactory proof of this. Ruska *et al.* (1952) described how the dense bodies may grow to abnormal sizes in old cultures, and we have observed similar forms (Pl. 3, fig. 13). However, this is rarely seen in the course of normal development, and it seems more likely that this swelling is another sign of the arrest of normal growth, comparable to the excessive vacuolization that is also seen. Werner (1951) believed that in some strains dense bodies may be absorbed as growth reaches its peak; however, this appearance may be due to the rapid reproduction of rods without an accompanying multiplication of dense bodies, with the result that young rods may have only one polar body or two extremely small ones (Pl. 2, fig. 9).

Lembke (1947) showed that dense bodies may be spontaneously liberated from old cultures or produced by mechanical disintegration, but they were not viable under these conditions. Some of our electron micrographs also suggest that dense bodies are liberated (Pl. 4, fig. 17). In mycelial cultures lysis is frequently accompanied by the appearance of granular forms. Microrods containing two dense bodies (Pl. 5, fig. 24) are also seen associated with disintegrating mycelial filaments, and there is evidence from phase-contrast studies (Brieger & Glauert, 1952) that these elements can germinate on removal to a fresh medium and produce a new generation of rods.

We wish to express our thanks to Mr R. W. Horne and Mr H. E. Pearson for their help in taking the electron micrographs. We are also grateful to Dr Dobson, of the Ministry of Agriculture's Research Station, Weybridge, for supplying the material from which the avian strains were recovered. Dr Steenken of the Trudeau Laboratory, Saranac Lake, kindly supplied us with the Sheard strain.

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EXPLANATION OF PLATES

Plate 1

Micro-cultures of *Mycobacterium avium* photographed with a Siemen's electron microscope operating at 70 kV. Some preparations shadowed at 30° with gold-palladium.

Fig. 1. Starting preparation from an 18-day old culture. Short rods with dense polar bodies and structureless cytoplasm. Bone strain, shadowed, × 12,500.

Fig. 2*a–d*. Impression prints taken after 20 hr. (*a* and *b*) or 48 hr. (*c* and *d*) growth on Löwenstein's medium. Discrete cellular units within elongating filaments. (*a*) Fn strain, × 13,000. (*b*) Fn strain, × 12,100. (*c*) Fn strain, shadowed, × 11,200. (*d*) Sheard strain, × 13,000.

Fig. 3. Impression print taken after 2 days growth on Löwenstein's medium. Retraction of cellular units. Fn strain, shadowed, × 12,800.

Fig. 4. 3 days growth on electron microscope grid on Löwenstein's medium. Filament segmenting. Dobson strain, shadowed, × 17,000.

Plate 2

Comparative phase-contrast and electron photographs of avian tubercle bacilli in impression prints from Löwenstein's medium.

Fig. 5. 20 hr. Apparently mycelial arrangement of filaments. Sheard strain. Phase-contrast microscope, × 1500.

Fig. 6. Electron microgram of group of filaments indicated in fig. 5. Electron microscope, × 12,100.

Fig. 7. 40 hr. Massive production of rods from filaments. Sheard strain. Phase-contrast microscope, × 4000.

Fig. 8. 20 hr. Corresponding phase in electron microscope. Sheard strain, × 12,600.

Fig. 9. 8 days. Completion of cycle. New generation of rods. Fn strain. Electron microscope. Shadowed, × 12,400.

Plate 3

Electron micrograms of avian tubercle bacilli showing vacuolization of the filaments and variations in dense bodies.

Fig. 10. 48 hr. growth on Löwenstein's medium removed by stripping with collodion film. Vacuolized filaments. AF strain, × 22,000.

Fig. 11*a*. Impression print from 4 days growth on Löwenstein's medium. Vacuolized filaments. Fn strain, shadowed, × 12,400.

Fig. 11*b*. 8 days growth on electron microscope grid on Löwenstein's medium. Vacuolized filaments. Fn strain, shadowed, × 12,600.

Fig. 12. Impression print from 6 days growth on Löwenstein's medium. Filaments packed with vacuolized units. × 12,400.

Fig. 13. 14 days growth on electron microscope grid on Löwenstein's medium. Swollen dense bodies. Fn strain, × 12,400.

Fig. 14. 14 days growth on electron microscope grid on Löwenstein's medium. Vacuolated dense bodies. Fn strain, shadowed, × 12,500.

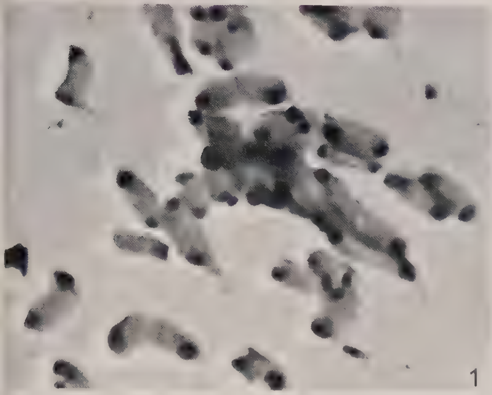
Plate 4

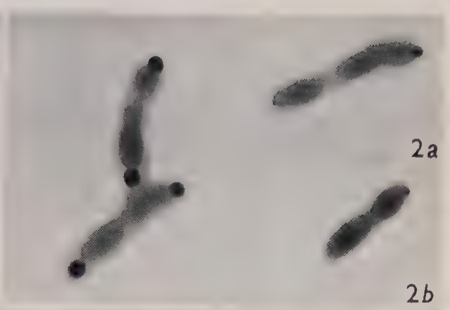
Electron micrograms showing variations in dense bodies

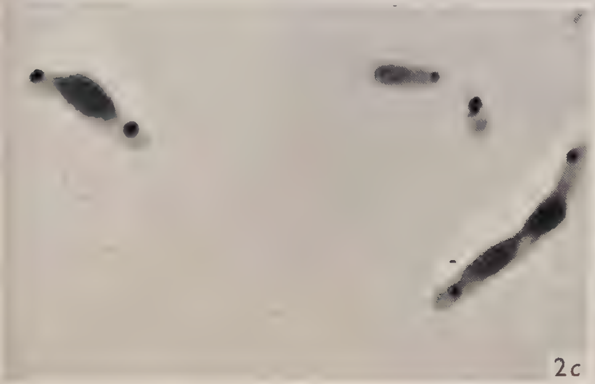
Fig. 15. 70 hr. growth of *Mycobacterium phlei* on electron microscope grid on embryo extract. Dense bodies with lighter areas. Shadowed, × 12,500.

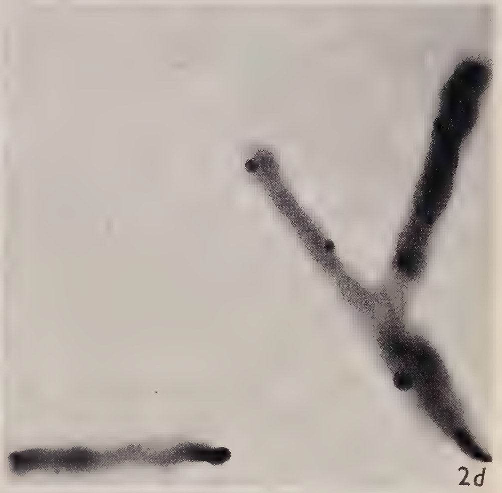
Fig. 16. 14 days growth of avian tubercle bacilli on electron microscope grid on Löwenstein's medium. Micro forms of dense bodies in proliferating filaments. Fn strain, × 17,000.

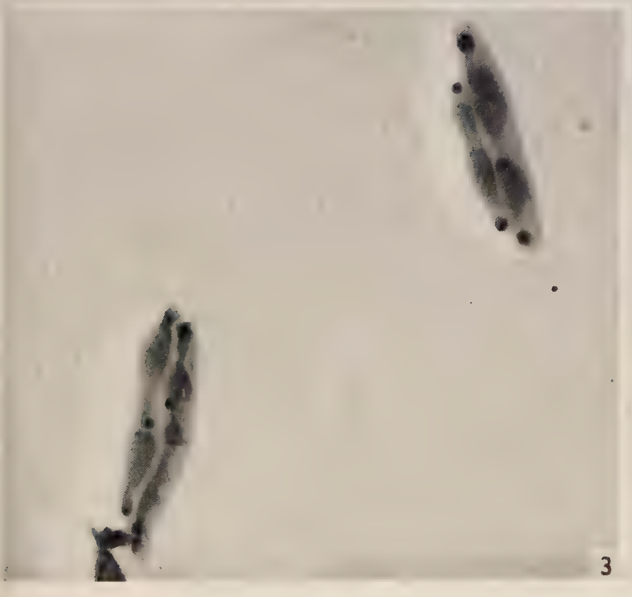
Fig. 17. 8 days growth of avian tubercle bacilli on electron microscope grid on Löwenstein's medium. Masses of dense bodies in transparent filaments. Fn strain, × 12,300.

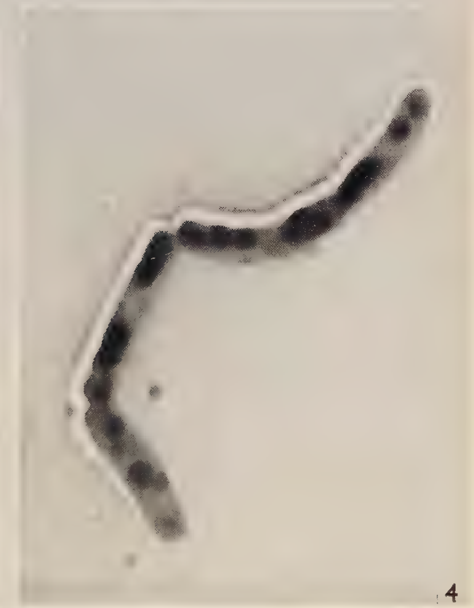












E. M. Brieger, V. E. Cosslett & A. M. Glauert—Electron microscopy of avian tubercle bacilli. Plate 1

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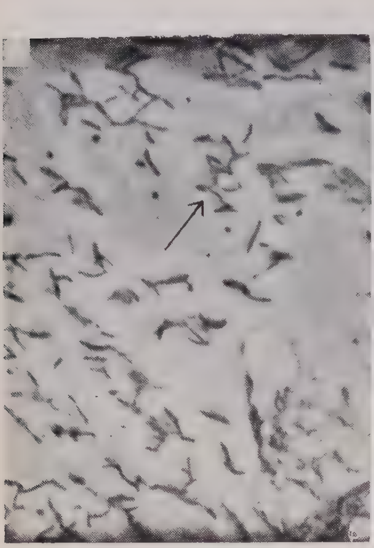

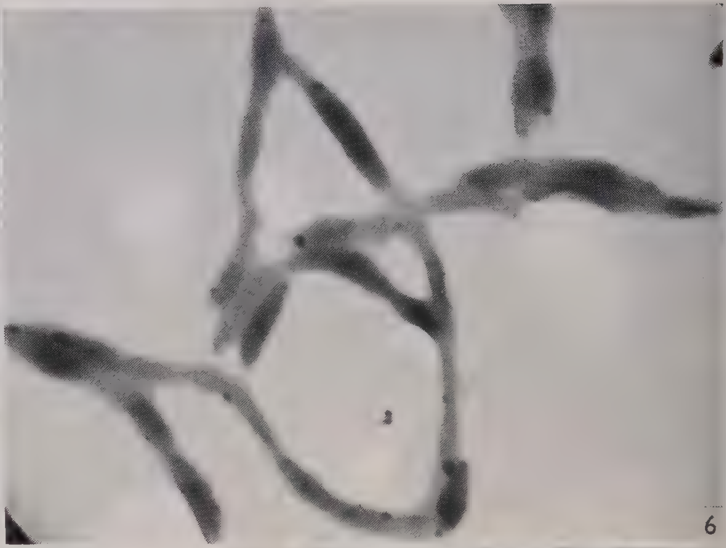

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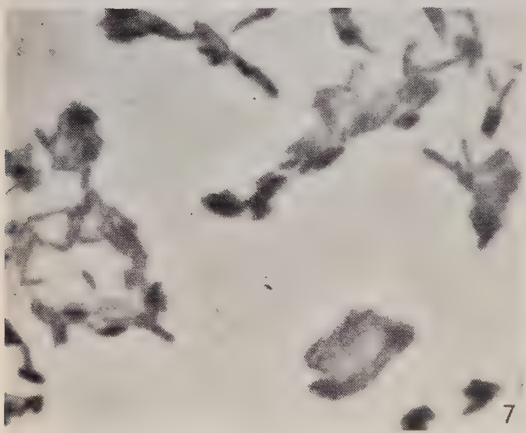

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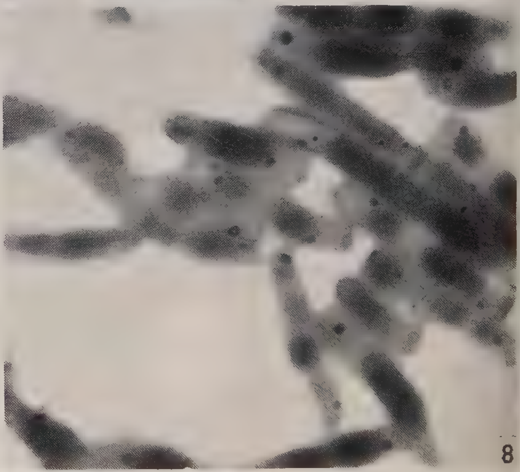

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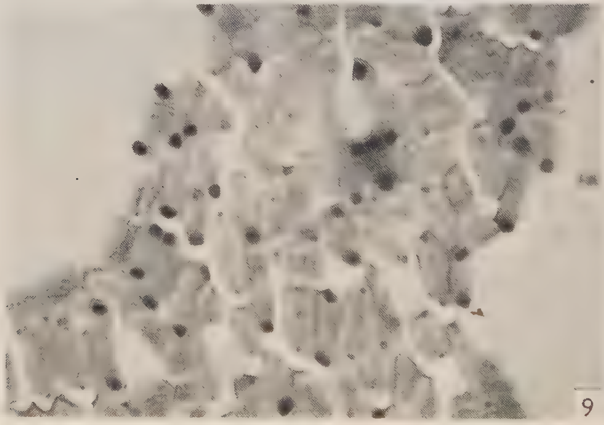

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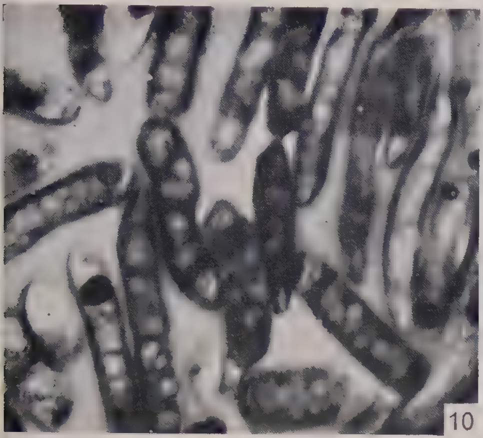
E. M. Brieger, V. E. Cosslett & A. M. Glauert—Electron microscopy of avian tubercle bacilli. Plate 2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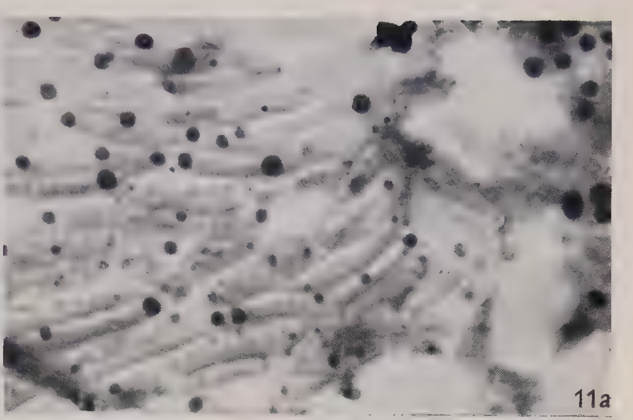
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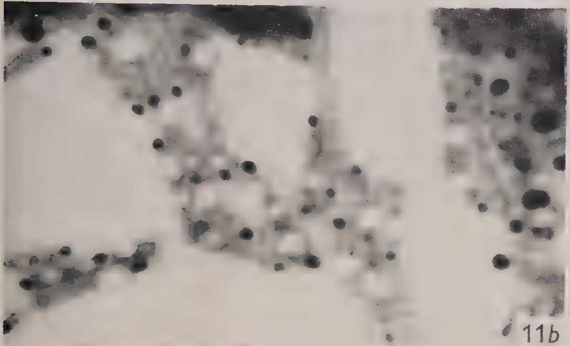
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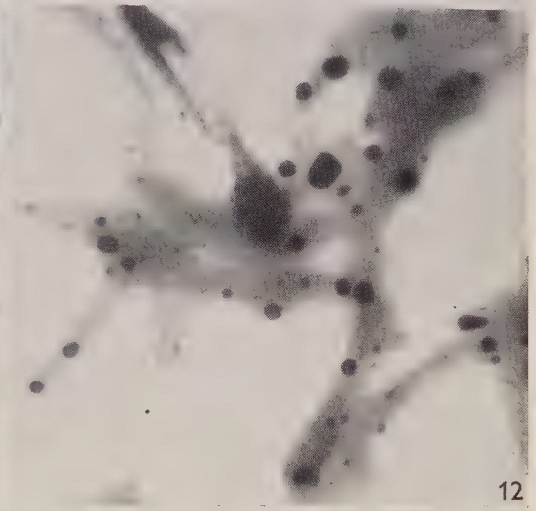
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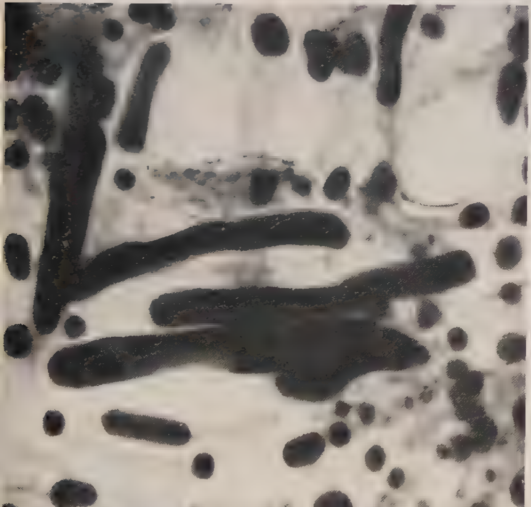
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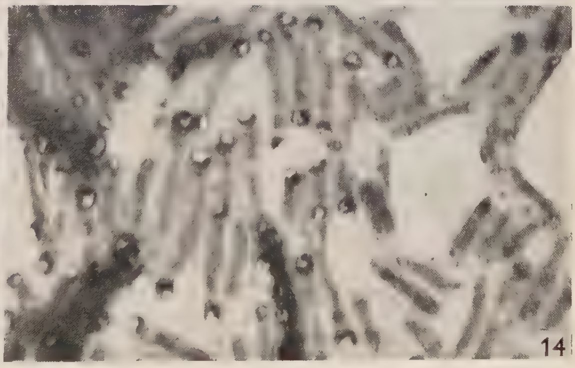
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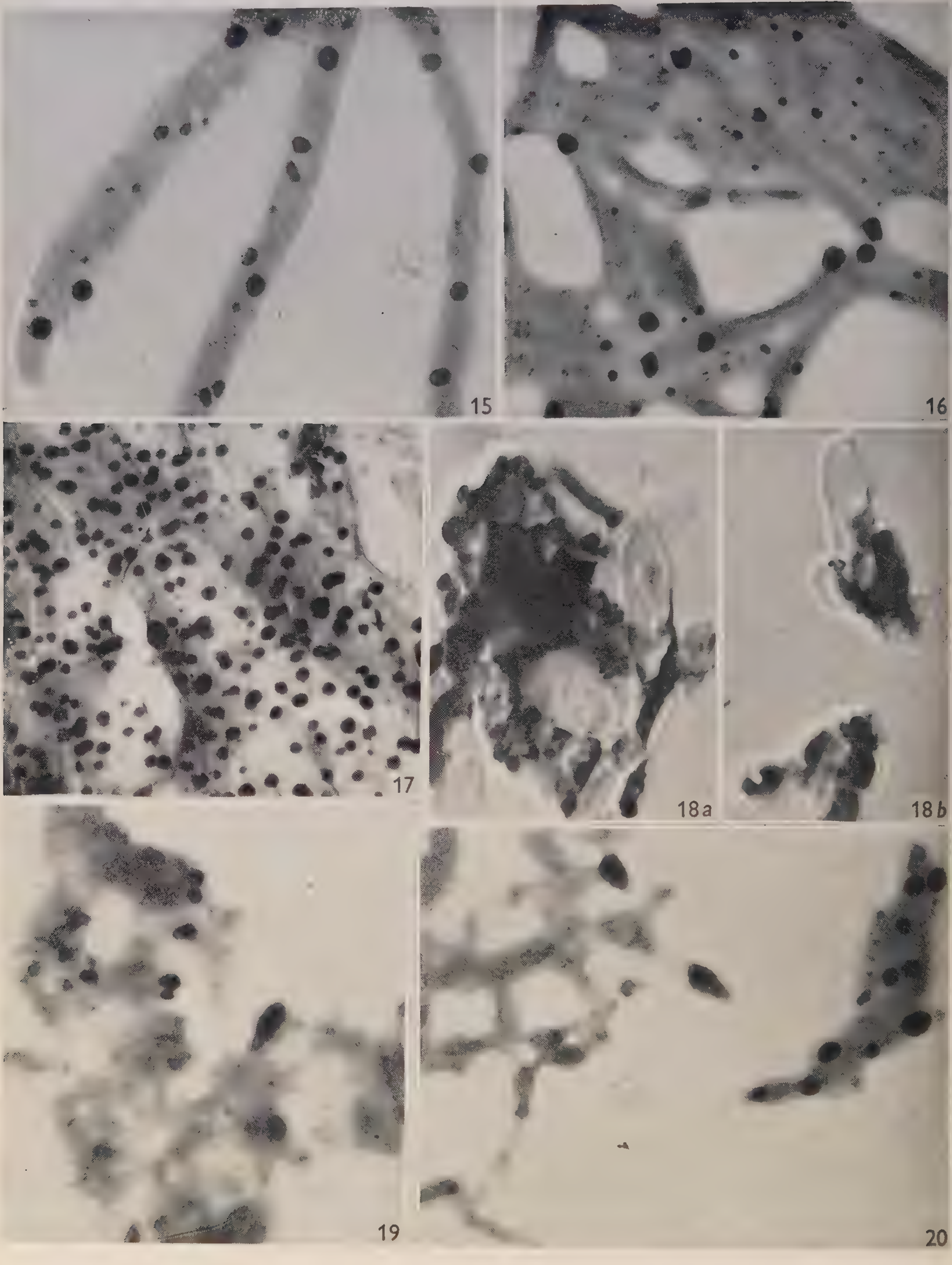
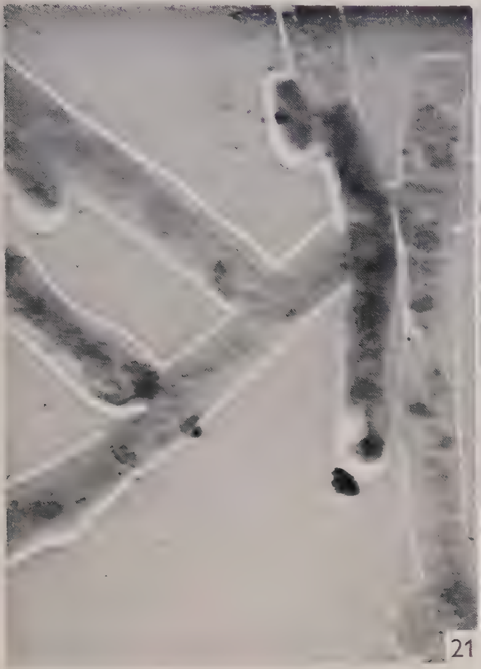
E. M. Brieger, V. E. Cosslett & A. M. Glauert -Electron microscopy of avian tubercle bacilli. Plate 3



E. M. Brieger, V. E. Cosslett & A. M. Glauert—Electron microscopy of avian tubercle bacilli. Plate 4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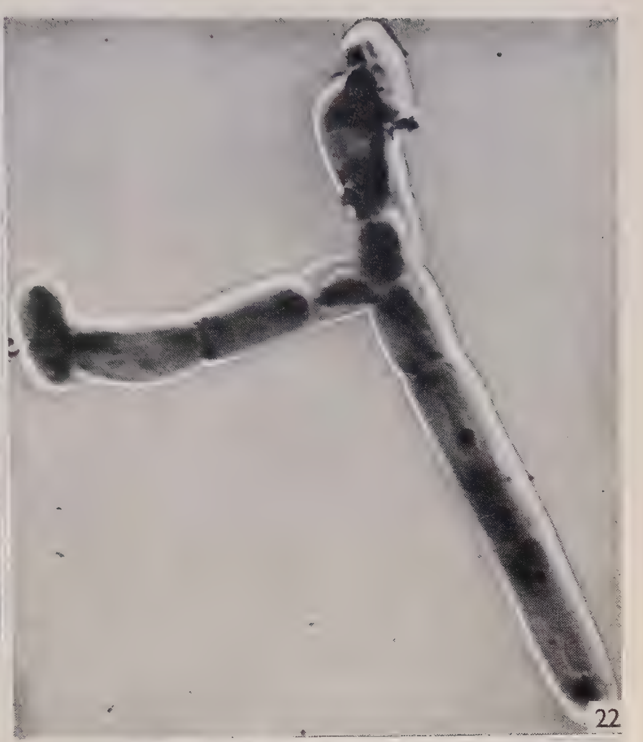
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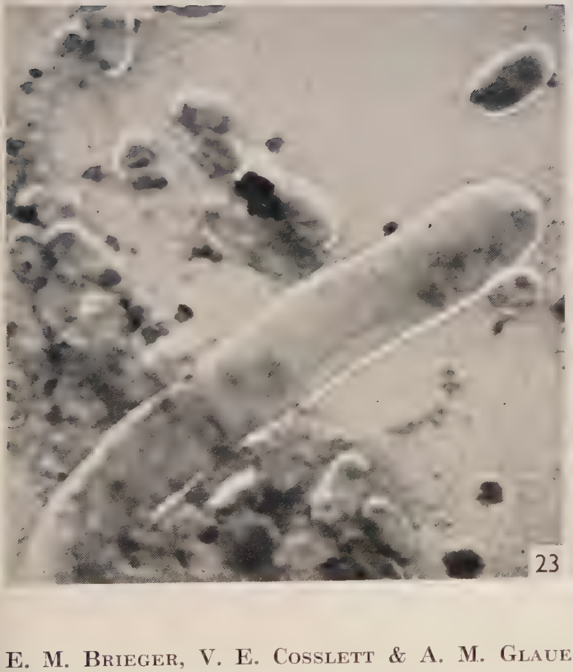
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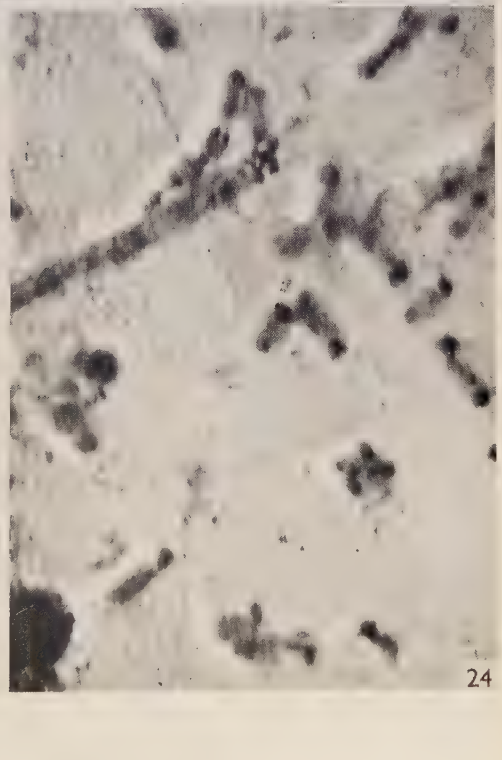
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E. M. Brieger, V. E. Cosslett & A. M. Glauert—Electron microscopy of avian tubercle bacilli. Plate 5

Electron micrograms of development in old cultures of avian tubercle bacilli.

Fig. 18*a*, *b*. Starting preparation of 13-month old culture. Rods merged in clumps and empty sheaths. Fn strain, shadowed, × 13,000.

Fig. 19. 2 days growth on electron microscope grid on Löwenstein's medium. Amorphous clump containing dense bodies. Fn strain, × 12,600.

Fig. 20. 4 days growth on electron microscope grid on Löwenstein's medium. Pear-shaped bodies resembling intracellular units in mycelial mesh. Fn strain, × 12,600.

PLATE 5

Electron micrograms of mycelial forms of avian tubercle bacilli. Material taken from a 7-week old culture of the Fn strain.

Fig. 21. Mycelium with transparent branches and associated micro-rods. Shadowed, × 18,000.

Fig. 22. Segmenting, branched filament. Shadowed, × 20,000.

Fig. 23. Lysis of mycelial filament. Associated granular elements. Shadowed, × 15,000.

Fig. 24. Disintegrating mycelial filament and bipolar micro-rods. Shadowed, × 12,000.

(*Received 7 September* 1953)

Few, A. V. (1954). *J. gen. Microbiol.* **10**, 304–308

Electron Microscopy of Disrupted Bacteria treated with Polymyxin E

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SUMMARY: Mechanically disrupted cells of polymyxin E sensitive strains of *Pseudomonas denitrificans* and *Escherichia coli* and a resistant strain of *Staphylococcus aureus* were studied in the electron microscope. The occurrence of a secondary layer or membrane was observed within the outer cell walls derived from *Ps. denitrificans* and *E. coli*; a similar structure was not observed in cell walls prepared from *Staph. aureus*. Treatment of the disrupted cells of *Ps. denitrificans* and *E. coli* with polymyxin E resulted in marked morphological changes, whilst similar exposure of *Staph. aureus* cell walls to the antibiotic produced no significant changes in their morphology.

From studies on the absorption of polymyxin E by intact bacteria and by bacterial cell-wall preparations, it has been shown that polymyxin-sensitive bacteria and cell walls are capable of rapidly absorbing much greater quantities of the antibiotic than similar preparations of resistant bacteria (Few & Schulman, 1953*a*, *b*). The differences in absorption behaviour were especially marked at low concentrations of polymyxin in the supernatant. As a result of the absorption of polymyxin by sensitive bacteria cell solutes were released by the organisms into the supernatant fluids, and it was suggested that polymyxin caused a disorganization of the structure responsible for osmotic regulation in sensitive bacteria.

It was considered that a study, by means of the electron microscope, of the action of the polymyxin E upon bacterial cell-wall preparations might yield direct evidence of cellular damage. The electron microscope studies of Mitchell & Crowe (1947) first demonstrated cytolytic damage caused by a bactericidal agent. Further investigations by Dyar (1947), by Meisel & Umanskaya (1949) and by Salton, Horne & Cosslett (1951) on the action of detergents of the quarternary ammonium type upon bacterial and yeast cells, demonstrated that in detergent-treated cells the cytoplasm contracted away from the cell envelope. Using intact *Pseudomonas aeruginosa*, Newton (1953) demonstrated that treatment of the organisms with polymyxin caused a leakage of cell solutes and was accompanied by changes in the morphology of the cells. The above authors observed changes in the morphology of intact cells when exposed to the bactericidal agents. The presence of electron-dense cytoplasmic material can cause difficulty in the observation of possible cell-wall changes brought about by the bactericidal agents; only a thin layer of the cell wall at the periphery of the cells can be seen clearly. With the exception of the work of Salton (1952) on the action of lysozyme upon cell walls derived from *Micrococcus lysodeikticus*, there appears to be little published data on electron microscope observations of the action of bactericidal agents upon isolated bacterial cell walls. The present paper is concerned with the morphological changes brought about by the absorption of polymyxin E by preparations of disrupted bacteria derived from sensitive strains of *Ps. denitrificans* and *Escherichia coli*, and those obtained from a resistant strain of *Staphylococcus aureus*.

METHODS

Preparation of disrupted bacteria

The three organisms investigated, *Ps. denitrificans*, *E. coli* and *Staph. aureus*, were of the same strains as those used previously (Few & Schulman, 1953*a*, *b*). Bacterial cells, obtained from 16 hr. cultures grown on a beef tryptic digest agar medium, were thrice washed with 0·01 M-phosphate buffer (pH 6·3). The cells were resuspended to *c*. 10 mg. dry wt./ml. in the buffer solution. Nine ml. of the bacterial suspensions were shaken in a Mickle disintegrator with an equal volume of no. 12 glass ballotini beads (English Glass Co., Ltd., Empire Road, Leicester). The shaking process was allowed to continue until most of the cells had been disrupted; a 5 min. shaking period was generally sufficient to cause the rupture of *c*. 95 % of the cells. The preparations were subjected to differential centrifugation to remove intact bacteria and debris.

Preparation of specimens for the electron microscope

Suspensions of the disintegrated bacteria at *c*. 0·15 mg. dry wt./ml. in 0·01 M-phosphate buffer (pH 6·3) were treated with a range of polymyxin E concentrations so chosen as to give both complete and incomplete saturation of the absorbing sites with the antibiotic. Samples of untreated and polymyxin E-treated suspensions were incubated at 25° for 20 min. The disintegrated bacteria were then centrifuged and resuspended in distilled water after the supernatant solutions had been removed. Drops of these distilled-water suspensions were placed on copper electron microscope specimen grids which had been covered with a thin nitro-cellulose film. Samples of the intact bacteria were also mounted in the same fashion. The specimens were dried in a desiccator over phosphorus pentoxide under reduced pressure. They were then shadowed with gold + palladium alloy (60 % + 40 %) at an angle of 30° to the plane of the specimen grid. A large number of mounted specimens were examined in a Siemens electron microscope, generally at direct magnifications of × 12,000–15,000.

RESULTS

Plate 1, fig. 1, Pl. 2, fig. 5, and Pl. 3, fig. 9, show the appearance of intact cells of *Ps. denitrificans*, *E. coli* and *Staph. aureus*. In all cases the cytoplasm has contracted away from the cell wall, which can be seen as a layer of uniform thickness. With the exception of *Staph. aureus* the intact bacteria cast very little shadow and seemed to lie flat on the supporting nitrocellulose film.

Plate 1, fig. 2, shows a typical preparation of disintegrated cells of *Ps. denitrificans*; the electron-dense cytoplasm has been removed, leaving the deflated cell walls. This preparation shows evidence of a duplex structure, consisting of an outer envelope or cell wall, together with a secondary inner layer which has contracted away from the outer cell wall. The uniform thickness of the inner layer suggests that it is not residual cell cytoplasm which, if present, generally occurs as irregular patches of electron-dense material. This inner layer is not complete in all the disrupted cells of *Ps. denitrificans*, and it appears that the layer can be readily detached from the outer cell wall.

The treatment of these disrupted bacteria with polymyxin E produced marked morphological changes. Pl. 1, figs. 3 and 4, show preparations which were exposed to 20 and 500 μg. polymyxin E/ml., respectively. The lower concentration gave rise to an absorption by the disrupted bacteria, which corresponded to the amount absorbed by these structures in the intact organisms when the cells were treated with just sufficient polymyxin E to give *c.* 99% mortality in 20 min. at 25°. The higher concentration used gave rise to complete saturation of the disrupted bacteria with the antibiotic, and corresponded to complete killing of the intact bacteria. The exposure of these preparations to polymyxin E resulted in a swelling of the bacterial structures. This swelling did not take place in a uniform manner, but resulted in the production of a mosaic pattern which is most clearly seen in Pl. 1, fig. 4.

The appearance of disrupted cells of *E. coli* (Pl. 2, fig. 6) is similar to that of *Ps. denitrificans*. The treatment of the disrupted cells of *E. coli* with polymyxin E also caused a disorganization of the cellular material. Pl. 2, figs. 7 and 8, show the appearance of these disrupted bacteria after exposure to 10 and 200 μg. polymyxin E/ml., respectively. Pl. 3, fig. 10, shows cell walls derived from polymyxin E-resistant cells of *Staph. aureus*. Pl. 3, figs. 11 and 12, show the appearance of these cell walls after they were treated with 20 and 200 μg. polymyxin E/ml. respectively. The examination of large numbers of disrupted *Staph. aureus* gave no indication of a duplex structure for these disrupted bacteria. Moreover, treatment of these preparations with polymyxin E did not result in any significant changes in appearance similar to those obtained with disrupted cells of *Ps. denitrificans* or *E. coli*.

DISCUSSION

By careful rupture of cells of *Ps. denitrificans* and *E. coli* it has been shown that the resulting disrupted cells possess a duplex structure, consisting of an outer cell wall and an inner secondary layer. It is evident that, whilst this inner layer possesses a certain structural rigidity, it is not so robust as the outer cell wall. Indeed prolonged shaking of the bacterial suspensions in the Mickle disintegrator produces from these organisms cell walls which are devoid of the secondary inner layer. The electron micrographs of partially autolysed cells of *Proteus vulgaris* and *E. coli*, obtained by Houwink & van Iterson (1950), clearly show the presence of a secondary layer or membrane within the cell wall of the organisms. Evidence of a similar layer can be seen at the periphery of the intact cell of *Ps. denitrificans* at the base of Pl. 1, fig. 1, of the present investigation.

Indirect evidence for the existence of this layer was also given by Salton & Horne (1951), who studied methods for the preparation of bacterial cell walls by the injection of bacterial suspensions into hot water. These authors noted that the cell cytoplasm, which had been removed from the cell walls of rod-shaped organisms, retained the original shape of the organisms. They suggested that this effect might either be due to the presence of another 'membrane' enclosing the cytoplasm or was caused by the heat coagulation of the cytoplasm, or by a combination of both. The results obtained in the present investigation provide strong support for the existence of an inner membrane within the cell walls of *Ps. denitrificans* and *E. coli*. From the work of Few & Schulman (1953*a*, *b*) and from the present investigations, it is evident that the permeability alterations brought about by the exposure of sensitive intact bacteria to polymyxin E, and which result in the leakage of cell solutes, can be correlated with morphological changes observed with polymyxin E-treated disrupted bacteria. It is tempting to suggest that the primary site of attack of this antibiotic is at the inner layer of these duplex structures, and that this inner layer or membrane is closely associated with osmotic-pressure regulation in the intact cell. The apparent absence of this membrane from cell-wall preparations of the polymyxin-resistant *Staph. aureus*, and the lack of any pronounced morphological changes when these preparations are exposed to the antibiotic, would perhaps support this view.

The author wishes to thank the Medical Research Council for a personal grant, Dr J. H. Schulman, O.B.E., and Dr V. E. Cosslett for helpful discussions, and Mr H. Pearson and Miss E. Green for taking the electron micrographs.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Intact cells of *Pseudomonas denitrificans*. × 20,000.

Fig. 2. Disrupted cells of *Ps. denitrificans*. Note the presence of an inner uniform layer within the cell wall. × 20,000.

Fig. 3. Disrupted cells of *Ps. denitrificans* after treatment with 20 μg. polymyxin E/ml. for 20 min. at 25°. × 20,000.

Fig. 4. Disrupted cells of *Ps. denitrificans* after treatment with 500 μg. polymyxin E/ml. for 20 min. at 25°. × 20,000.

Plate 2

Fig. 5. Intact cells of *Escherichia coli*. 20,000.

Fig. 6. Disrupted cells of *E. coli*. Note the presence of an inner layer within the cell wall. × 20,000.

Fig. 7. Disrupted cells of *E. coli* after treatment with 10 μg. polymyxin E/ml. for 20 min. at 25°. × 20,000.

Fig. 8. Disrupted cells of *E. coli* after treatment with 200 μg. polymyxin E/ml. for 20 min. at 25°. × 20,000.

Plate 3

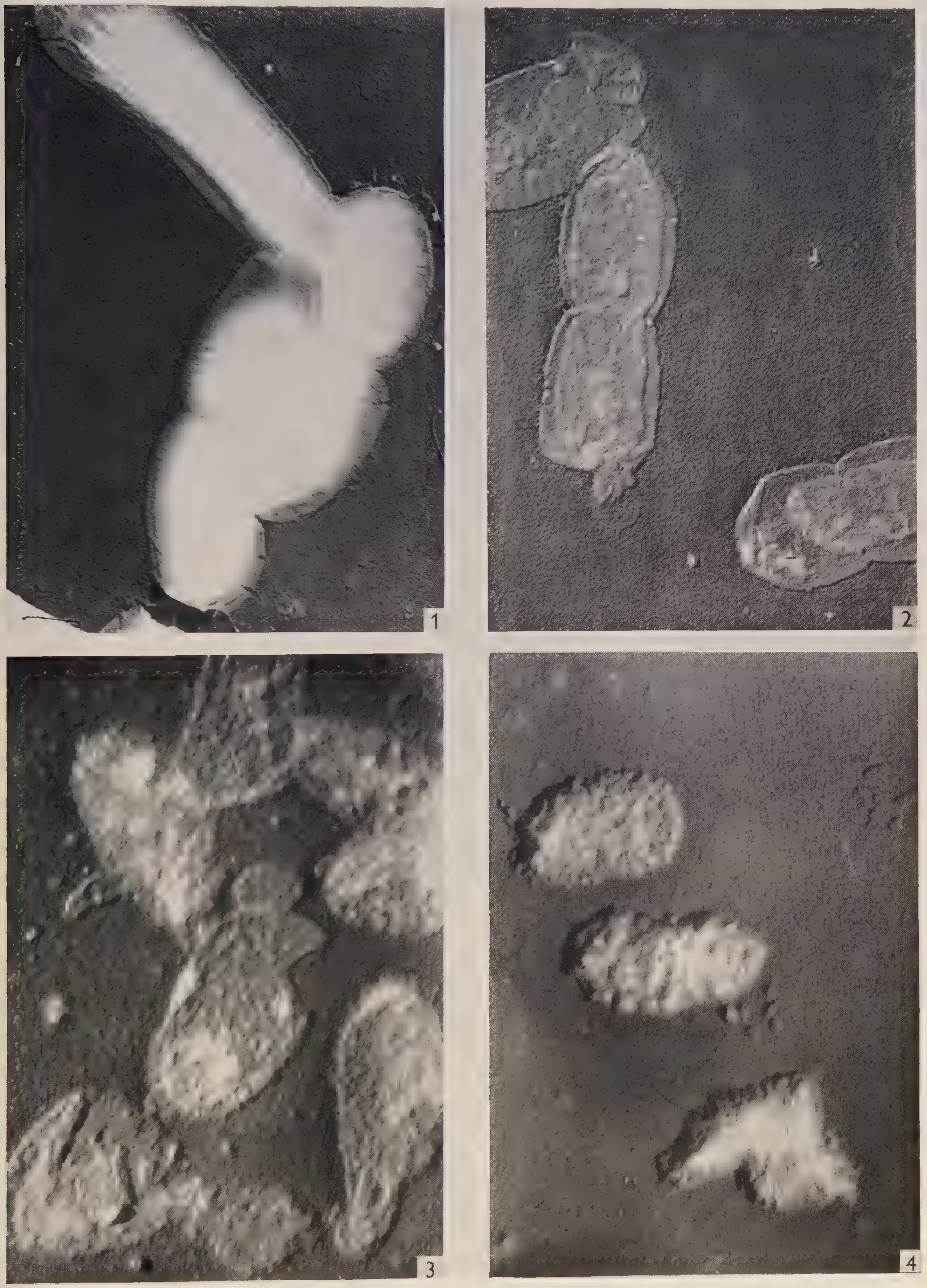
Fig. 9. Intact cells of *Staphylococcus aureus*. × 20,000.

Fig. 10. Cell walls of *Staph. aureus*. × 20,000.

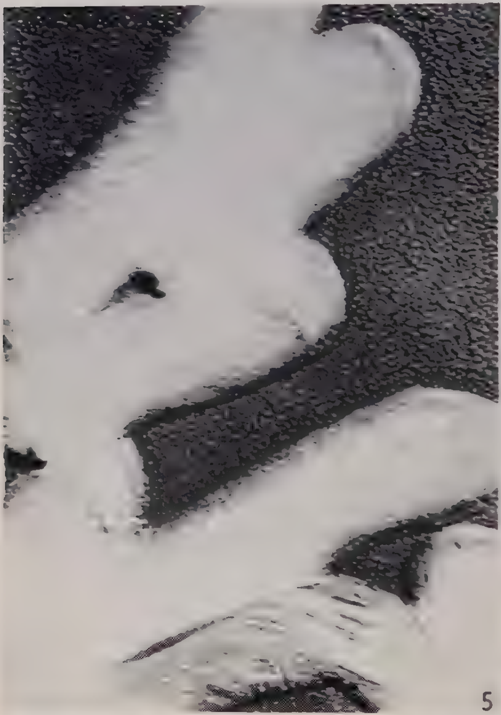
Fig. 11. Cell walls of *Staph. aureus* after treatment with 20 μg. polymyxin E/ml. for 20 min. at 25°. × 20,000.

Fig. 12. Cell walls of *Staph. aureus* after treatment with 200 μg. polymyxin E/ml. for 20 min. at 25°. × 20,000.

(*Received* 25 *September* 1953)



A. V. FEW—POLYMYXIN-TREATED DISRUPTED BACTERIA. PLATE 1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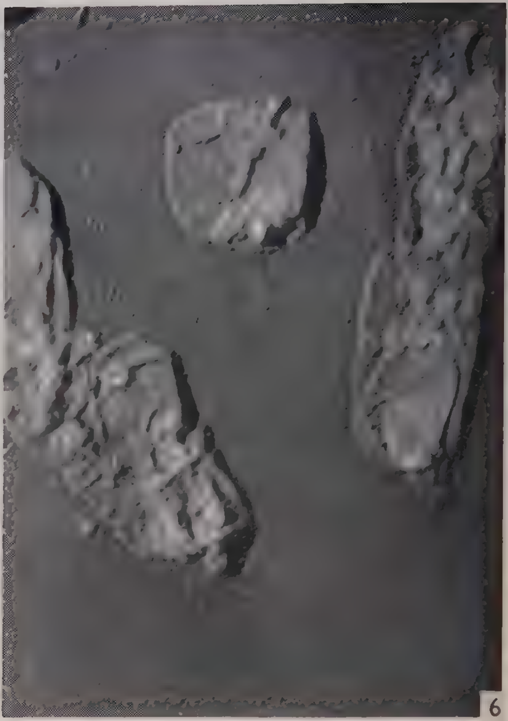
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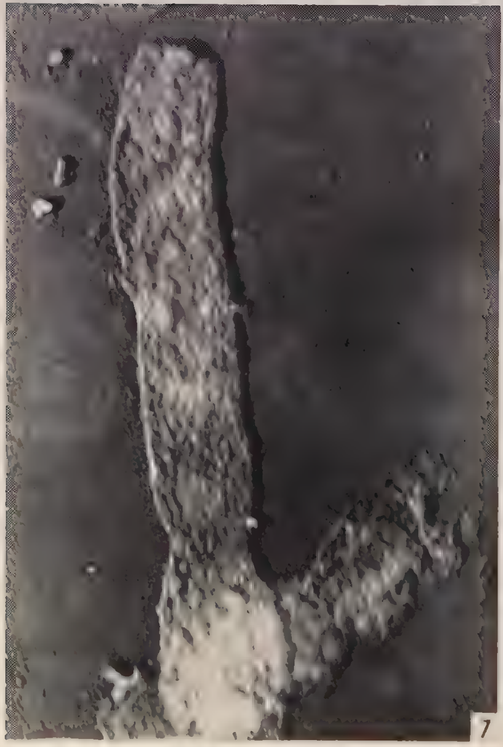
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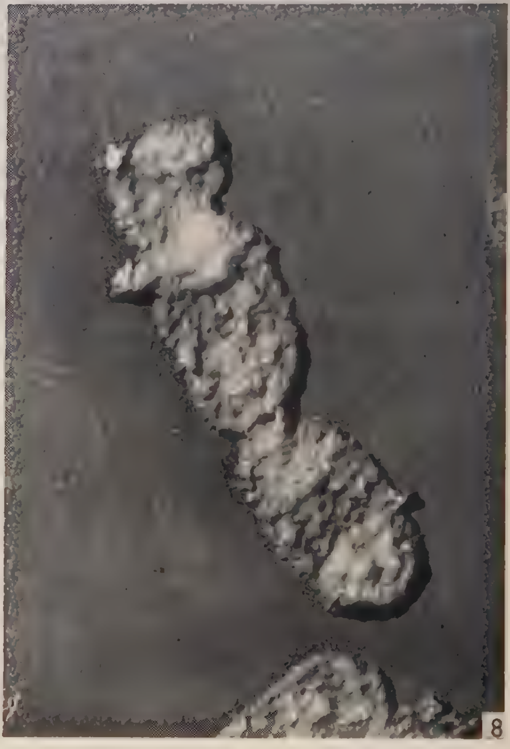
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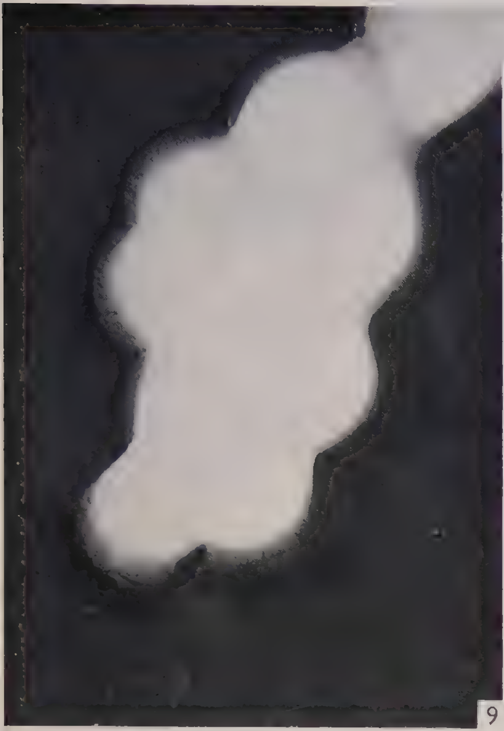
A. V. FEW—POLYMYXIN-TREATED DISRUPTED BACTERIA. PLATE 2



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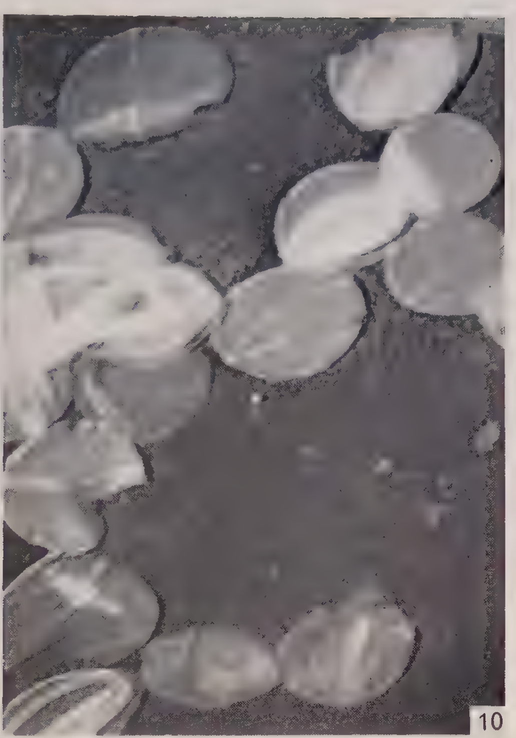

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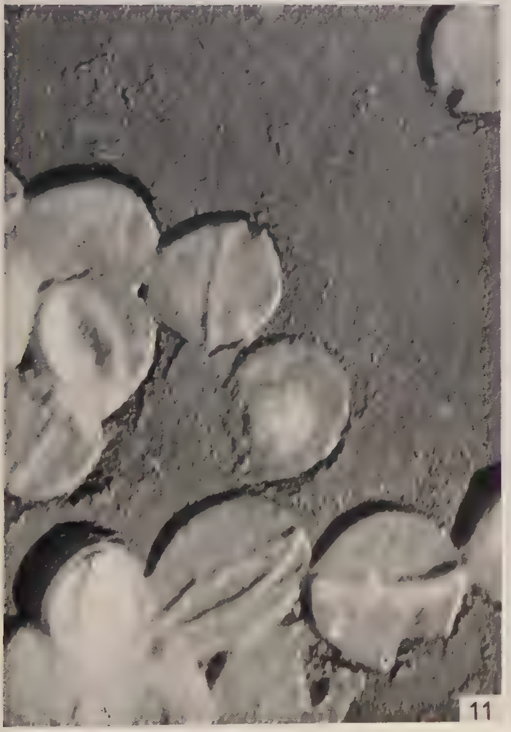

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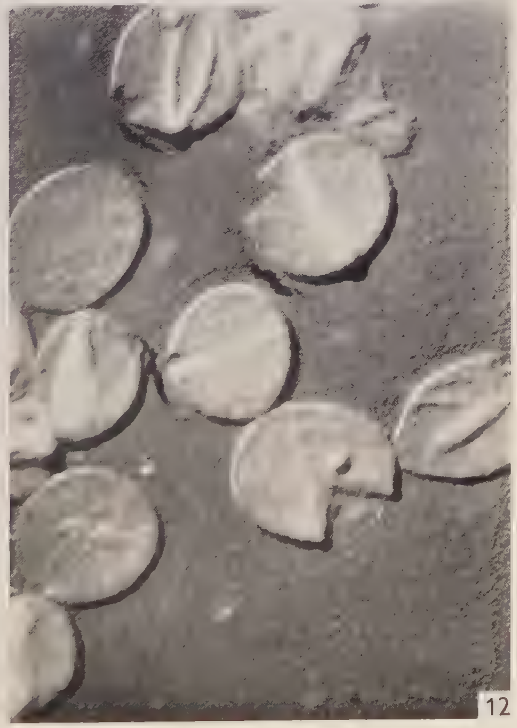

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A. V. Few—Polymyxin-treated disrupted bacteria. Plate 3

FAIRBROTHER, R. W., PARKER, L. & EATON, B. R. (1954). *J. gen. Microbiol.* **10**, 309–316

The Stability of Penicillinase-producing Strains of *Staphylococcus aureus*

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SUMMARY: Penicillin-sensitive and penicillin-resistant colonies of *Staphylococcus aureus*, belonging to the same phage pattern, were found in six out of 200 apparently resistant cultures isolated from infective processes. The *in vitro* instability of penicillinase-producing strains of *Staph. aureus* was confirmed. It was found that growth with *Streptococcus haemolyticus* or in the presence of other antibiotics did not greatly influence the natural development of sensitive variants. This process, however, tended to be accelerated by growth at 44°, although some strains did not yield any sensitive variants in spite of prolonged incubation. Some inherited factor is considered to be primarily responsible for the production of penicillinase.

Two different types of resistance to penicillin are exhibited by *Staphylococcus aureus*. One type can be readily induced *in vitro* by serial subculture in increasing concentrations of the antibiotic; this change is reversible and is not associated with the formation of penicillinase. The other type has only been obtained under natural conditions; it tends to be permanent and is due to the production of penicillinase. These are quite distinct phenomena and require individual consideration.

Penicillinase-producing strains of *Staph. aureus* are being isolated from infected lesions with increasing frequency, especially in hospitals, and they present a serious clinical, as well as an important fundamental, problem. The origin of these strains has not been determined; both genetic and environmental factors have been incriminated, but conclusive evidence of their particular role has not been obtained. The only apparent difference between these resistant strains and sensitive strains is the production of penicillinase, which some workers consider to be a permanent characteristic of naturally occurring resistant strains. Recent work, however, indicates that these strains can give rise to sensitive variants.

Voureka (1948) obtained sensitive organisms by growing resistant strains of *Staph. aureus* in mixed culture with *Streptococcus haemolyticus* or other organisms; she suggested that this change was due to the activity of some substance produced by the associated organism. Workers at the Johns Hopkins University (Chandler, Davidson, Long & Monnier, 1951; Monnier & Schoenbach, 1951; Chandler & Goltz, 1952) reported a marked diminution in penicillinase production, with the appearance of sensitive variants, by growing penicillin-resistant staphylococci in the presence of other antibiotics. Barber (1949, 1953) found that old cultures of penicillin-resistant staphylococci did not always contain a homogeneous population; by repeated subculture over a long period, sensitive variants could often be isolated from the resistant strains. She therefore considered that any variation in sensitivity, which occurred during the various procedures, was due to the natural selection of sensitive variants and not to any specific action on the penicillinase system. There is thus no doubt that penicillinase-producing strains of *Staph. aureus* can produce sensitive variants, but the mechanism responsible for this change is uncertain. As this is a problem of fundamental interest, it was decided to carry out further investigations, particularly on (1) the natural occurrence of staphylococcal variants in infective material, and (2) methods of increasing the natural instability of penicillinase-producing strains of *Staph. aureus*.

METHODS

All strains of *Staph. aureus* used were coagulase-positive by the tube test and those tested fermented mannitol and liquefied gelatin.

Sensitivity tests were carried out by: (1) the dried disk and strip technique (Fairbrother & Martyn, 1951); (2) the serial dilution method, in which heavy inocula were used—1 drop (0·02 ml.) of a 24 hr. culture to 0·5 ml. of medium (*c.* 20 million organisms). Results in both methods were read after 18 hr. incubation.

Penicillinase tests. Agar plates were seeded uniformly with the Oxford strain of staphylococcus; sterile cylinders were placed on the medium and into them, first, 4 drops of an overnight broth culture of the test organism, and then 1 drop of penicillin solution (10 units/ml.) were pipetted; readings were made after overnight incubation. When this test failed to demonstrate the presence of penicillinase the organism was re-examined by a modification of the technique of Harper (1943). A suspension (0·5 ml.) in distilled water of the acetone and ether-treated cells was added to falling dilutions of penicillin in 0·5 ml. broth; the end-point was compared with a similar series of dilutions of penicillin to which distilled water only had been added. The presence of penicillinase is indicated by a difference in end-points.

Phage typing of the strains used was done at the Staphylococcal Reference Laboratory, Colindale.

RESULTS

The presence of resistant and sensitive strains of Staphyloccocus aureus *in the same lesion*

It is generally accepted that penicillinase-producing organisms cannot be produced *in vitro* from sensitive strains of *Staph. aureus*. It has, however, been suggested that penicillinase-producing mutants may arise from sensitive strains *in vivo* during treatment with penicillin; conclusive evidence to support this claim has not yet been produced. It is therefore surprising that few investigations on the occurrence of staphylococcal variants *in vivo* have been carried out. Barber & Whitehead (1949) examined individual colonies of cultures made from infected material and isolated sensitive and resistant strains of *Staph. aureus* from the same patients on eleven occasions. The strains were phage-typed but, as one or both of the pair was untypable in eight cases, the results were inconclusive; in the majority of cases the organisms appeared to belong to different types.

In routine work, sensitivity tests by the disk or similar technique are done either on the original material or, more frequently, on pure cultures prepared indiscriminately from the original plate. Under such conditions the detection of variants is difficult, owing to the production of penicillinase by resistant organisms and consequent inactivation of the test penicillin. As a general rule the presence of penicillin-sensitive variants in a resistant culture of *Staph. aureus* can only be detected by the examination of the individual colonies. Accordingly, discrete colonies (15–50) were selected at random from 200 original cultures of infected material, found to be resistant in the routine test, and examined by the strip technique.

Occasional sensitive variants were found on thirteen occasions. In two cases, only one sensitive colony was found out of totals of forty and fifty respectively. It is possible that the number of cultures with sensitive variants would have been greater if more colonies had been selected from the original plates, as fifty colonies represent only an extremely small sample of the whole culture. The variants were checked for coagulase-production, and the sensitivity of the sensitive and resistant organisms determined by the serial dilution method. The resistant strains were only inhibited by at least 100 units penicillin/ml. and produced penicillinase, whereas the sensitive strains were inhibited by $\frac{1}{8}$ unit or less penicillin/ml. and did not form penicillinase.

The resistant and sensitive strains, as well as the original culture, were phage-typed. In five of the thirteen mixed cultures of penicillin-sensitive and penicillin-resistant staphylococci the two strains were of different phage-patterns and, in two, one of each pair was untypable. These findings strongly suggest that, in these seven cases, the two strains originated from different sources.

In six of the thirteen mixed cultures, however, the two strains had the same phage-pattern and the organisms of each pair probably originated from the same parent strain. Only occasional sensitive colonies were isolated in each instance from the infected material, which was not, however, examined in the initial stages of the infection. There was, therefore, no conclusive evidence to indicate the sensitivity to penicillin of the original infecting strain, or the mechanism by which the variants developed *in vivo*.

In vitro *development of sensitive variants from penicillinase-producing* Staphylococcus aureus

The natural instability of some penicillinase-producing strains of *Staph. aureus* was originally demonstrated by Barber (1949), who considered that, apart from repeated subculture, there was no way of accelerating this natural tendency. Growth with other organisms or in the presence of other antibiotics made no significant difference. In view of other claims, the *in vitro* development of sensitive variants from resistant organisms was investigated by three different methods: growth with *Strep. haemolyticus*; growth in the presence of other antibiotics; growth at 44°.

Growth in the presence of Streptococcus haemolyticus. Following the technique of Voureka (1948), penicillinase-producing strains of *Staph. aureus* were grown in mixed culture with a penicillin-sensitive strain of *Strep. haemolyticus* (group A). Twenty resistant strains of *Staph. aureus* were examined and seven different strains of *Strep. haemolyticus* used. After incubation at 37° for 24 hr., subcultures of the original and the mixed staphylococcal cultures were prepared on blood agar plates. After overnight incubation, ten to fifty colonies were tested for penicillin sensitivity by the strip technique. Eighteen of the twenty strains of *Staph. aureus* yielded only resistant colonies. From one strain, however, a few sensitive colonies were obtained, but these were isolated in similar proportions from both the original and treated cultures. The sensitive variant belonged to the same phage-type as the resistant organism. With another strain, one sensitive colony was isolated from the original culture but none was isolated from the treated culture, fifty colonies being examined in each case. These findings support the view of Barber that the development of sensitive variants from penicillin-resistant strains of *Staph. aureus* after mixed culture is due to the natural instability of these strains and not to the specific action of any substance produced by the other organisms.

Growth in the presence of other antibiotics. Using the technique of Chandler *et al.* (1951) strains of *Staph. aureus*, which were highly resistant to penicillin but sensitive to the other antibiotics, were grown in broth containing increasing concentrations of the other antibiotics. Sensitivity tests by the serial dilution method were done before and after the treatment (Table 1). In this series, alteration in resistance to penicillin was observed only after serial subculture in broth containing increasing concentrations of streptomycin (initial concentration 1 μg./ml. and increasing 64- to 32,000-fold), when four of eleven strains showed an increase in sensitivity to penicillin. This increased sensitivity was only slight in the case of three strains, from two of which a few sensitive colonies (one among twenty) were detected on colonial examination of cultures prepared both before and after streptomycin treatment. Subculture in the streptomycin broths had not affected their frequency. One resistant strain (Law), however, exhibited a marked transformation; after ten subcultures in

Table 1. *The effect of serial subcultivation in increasing concentrations of other antibiotics on penicillin resistance of* Staphylococcus aureus

			Change in resistance	
Antibiotic*	No. of strains tested	No. of subcultures	Increase to homologous antibiotic	Decrease to penicillin
Aureomycin	6	10 to 20	4- to 8-fold	No change
Terramycin	3	10 to 15	8- to 16-fold	No change
Chloramphenicol	1	10	4-fold	No change
Streptomycin	11	9 to 12	64- to 32,000-fold	7 = no change 3 = 4- to 16-fold 1 = 512-fold

* Aureomycin—initial concentration = 0·125 μg./ml. increasing to a maximum of 8 μg./ml. Terramycin—initial concentration = 0·5 μg./ml. increasing to a maximum of 8 μg./ml. Chloramphenicol—initial concentration = 8 μg./ml. increasing to 32 μg./ml. Streptomycin—initial concentration = 1 μg./ml. increasing to a maximum of 32,000 μg./ml.

the presence of streptomycin (final concentration 8192 μg./ml.), it became completely sensitive to penicillin. The original culture had proved to be unstable, as it yielded a few sensitive colonies, but a resistant colony had been selected for the test. After serial subculture in the presence of streptomycin, the resistance to which increased 32,000-fold, twenty out of twenty colonies were highly sensitive to penicillin. With a mass culture, the disk technique gave a wide clear zone and there was no production of penicillinase. The resistant and sensitive organisms had the same phage-type.

In view of these results, two more resistant colonies were selected from the original 'Law' culture and subjected to similar treatment. In both cases, the result was quite different. There was a 16,000-fold increase in resistance to streptomycin, but the sensitivity to penicillin was unaltered. Chandler & Goltz (1952) reported a similar inconsistency in the behaviour of a resistant strain of *Staph. aureus*. They found that one strain (strain 8) remained unchanged after transfer through aureomycin broth, and yet, in an earlier investigation, Chandler *et al.* (1951) had found that this strain permanently lost its capacity to produce penicillinase after a similar course of treatment.

These results indicate that the action of other antibiotics on penicillinase-producing strains of *Staph. aureus* is erratic and unpredictable. It seems more probable that the development of sensitive variants is due to the inherent instability of these strains rather than to any specific action of the other antibiotics on the penicillinase mechanism.

Growth at 44°. Since enzyme activity diminishes on increasing the temperature above the range of optimal activity, it was considered that, by incubation above 37°, the conversion of penicillinase-producing strains of *Staph. aureus* into sensitive variants might be accelerated. In consequence, serial subcultures in broth of resistant strains were incubated in a water-bath at 44°. This was found to be the highest temperature that would regularly permit active growth of these strains.

Resistant strains, freshly isolated from active infections, were selected for the trial. Subcultures of a resistant colony were prepared on blood agar or agar plates. If twenty out of twenty colonies were found to be resistant, a known resistant colony was replated and, if all tested colonies of the subculture proved to be resistant, one of these was subcultured into 10 ml. broth in a 1 oz. screw-capped bottle and incubated at 44°. At intervals usually of 1–3 days serial subcultures were made in broth by means of a large loop; at the same time plate cultures were generally incubated at 37° for sensitivity tests. Growth occurred readily in the early subcultures but tended to become slower and less marked in the later subcultures. Six different strains were investigated by this technique. In one case, a single-cell culture was also examined; in another, two separate colonies were tested and, with a third strain, a series of subcultures was also incubated at 37° under otherwise identical conditions. The results are given in Table 2.

With the 'L' strain sensitive variants were first isolated after thirteen transfers during 24 days, when seven out of sixty colonies proved to be sensitive. On further incubation, the number of sensitive colonies increased and, after twenty-three subcultures, only sensitive colonies were obtained. It is interesting to note that the original subculture, which had been incubating at 44° during this period, was tested on several occasions but yielded only one sensitive colony out of fifty-nine examined.

Table 2. *Development of sensitive variants from recently isolated resistant strains of* Staphylococcus aureus, *when serially subcultivated at* 44°. *Results of colony tests.*

	Number of subcultures				
	1–10	11–20	21–30	31–40	41+
Strain of *Staph. aureus*	Ratio sensitive/total colonies tested				
‘L’	0/36	15/90	40/63	—	—
‘W’	0/57	4/43	55/57	—	—
‘W’ (single coccus)	3/87	0/83	0/76	10/151	0/328
‘G’	0/50	0/50	0/60	0/100	0/690
‘LAW’ (1)	0/200	0/200	0/120	0/40	0/120
‘LAW’ (2)	2/200	0/200	0/120	0/40	0/80
‘R’	0/200	0/200	0/200	0/40	—
‘H’	8/77	49/116	56/56	—	—
‘H’ (37°)	0/75	0/84	12/69	—	—

The ‘W’ strain produced similar results. Sensitive colonies were first isolated after fifteen subcultures; these increased in number with subsequent transfers and, after twenty-four subcultures, only sensitive colonies were obtained. At this stage the original subculture, kept throughout at 44°, yielded only resistant colonies but, after continuous incubation for several months sensitive variants were eventually isolated. The results given by the ‘L’ and ‘W’ strains indicated that repeated subculture at 44° had a profound effect on penicillinase-producing organisms and suggested that the change in sensitivity might be an adaptation response to the higher temperature of incubation. In order to obtain further evidence of this change, a similar series of subcultures was made from a single-cell culture of the ‘W’ strain, which was made by Dr K. I. Johnstone, Leeds University. Seventy-seven subcultures were prepared during 7 months and only thirteen of 725 colonies proved to be sensitive to penicillin. These colonies were isolated at irregular intervals but were not detected after the 38th subculture.

With the ‘G’ strain 100 subcultures were made in 9 months and all tested colonies (850/850) proved resistant.

‘Law’ was the strain which gave anomalous results in the streptomycin experiments. Two resistant colonies were tested; one yielded only resistant colonies (680/680) during subcultivation, while the other yielded two sensitive colonies from 640 taken, both of which were isolated from the initial subculture.

With the ‘R’ strain thirty-two subcultures were made and all tested colonies (640/640) were resistant.

The ‘H’ strain was tested at 44° and 37°; a single resistant colony was selected and seeded first into the medium at 37° and then directly into that at 44°; thirty subcultures in each series were made during 10 weeks. Sensitive variants of this strain were more numerous and appeared more rapidly in the series incubated at 44°.

In all cases, whenever sensitive variants were isolated, the resistant and sensitive organisms were tested for coagulase and penicillinase, fermentation of mannitol, liquefaction of gelatin and phage-type. Invariably there was complete agreement except in the case of penicillinase-production, when the sensitive variants always gave negative results. Periodic examinations were also made on organisms which remained persistently resistant; these invariably behaved in the same manner as the parent strain. The sensitive variants tended to show definite colonial changes, becoming less pigmented and flatter, but these changes were not always associated with a change in sensitivity to penicillin.

DISCUSSION

These results confirm the view that cultures, and indeed individual colonies, of penicillinase-producing strains of *Staph. aureus* do not always contain a homogeneous population. Some strains exhibit a natural tendency to produce sensitive variants, the incidence of which is accelerated by repeated subcultivation at 44° but is not greatly affected by growth in the presence of a haemolytic streptococcus or another antibiotic.

These findings cannot readily be explained by the concept of physiological adaptation to the modified environment. Some form of mutational change seems a more probable explanation. It is therefore suggested that some inherited factor controls the production of penicillinase by naturally occurring resistant strains but, in view of the limited knowledge of bacterial genetics and the primitive character of the staphylococcus, it is doubtful if this factor should be termed a gene. Staphylococci propagate by fission, but it is probable that the bacterial cells do not always divide into two identical daughter cells. In consequence any factors originally present may be unevenly distributed among the progeny.

When resistant strains of *Staph. aureus* possess relatively large amounts of the penicillinase factor, the development of sensitive variants may not occur, even at 44°. In some cases, e.g. 'W' single-cell culture and 'LAW 2', a few sensitive variants may be produced erratically but these are unable to establish themselves, being overwhelmed by the resistant, penicillinase-producing organisms. On the other hand, strains possessing only a small proportion of this factor may readily produce sensitive variants, particularly if the environmental conditions are suitable. Growth at 44° probably accelerates the development of sensitive variants by retarding the rate of growth of the resistant organisms. The production of penicillinase is decreased but not inhibited by incubation at 44°. Penicillinase-producing strains of *Staph. aureus* are frequently unstable and, while the development of sensitive variants is probably dependent on inherited factors, this process can be accelerated by modifying the conditions of growth.

We wish to thank Dr R. E. O. Williams and Miss Joan E. Rippon for phage-typing the various strains of *Staph. aureus*, and Dr K. I. Johnstone for preparing the single-cell culture of the 'W' strain.

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(*Received* 29 *September* 1953)

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A Specific Cell-wall Reaction in *Bacillus* sp.

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SUMMARY: The cell wall of a bacillus, related to *Bacillus anthracis*, reacts specifically with the homologous antibody. This reaction can be made clearly visible by phase contrast when cell wall and cytoplasmic membrane are separated by mild lysozyme digestion, or when the cytoplasm is partially autolysed or digested with trypsin. The same antibody reacts with the transverse septa and polar bodies of the capsule, rendering them visible by phase contrast. These septa are in continuation of the cross walls of the cell, and sometimes give the appearance of division.

By these means the morphological locations of the chemically isolable fractions of serologically active bacterial extracts can be defined. These substances in the cell wall and in the septa and polar bodies of the capsule appear to be polysaccharide. The D-glutamic acid polypeptide element of the capsule does not occur in the cell wall.

A non-specific cell-wall reaction can be obtained with proteins at the appropriate values of pH, analogous to the non-specific capsular reaction previously reported.

Few observations have been made upon the visible effects of antibody on the bacterial cell wall. An increase in electron opacity and a decrease in clarity of outline of *Salmonella typhi* and *Bacillus subtilis,* after reaction with homologous antibody, was observed by Mudd & Anderson (1941). We have ourselves observed that non-capsulated bacteria appeared to increase in thickness, as seen by the phase-contrast microscope, after reaction with the homologous serum, which may be due to a precipitation of antibody on the cell-wall surface. We have shown that a morphologically and chemically complex structure can be demonstrated by these means in the capsule of *B. megaterium* and the related *Bacillus* M (Tomcsik, 1950, 1951; Tomcsik & Guex-Holzer, 1951). Whereas with anthrax D-glutamic polypeptide antibody a homogeneous capsular material was made visible by phase contrast, the appearance with homologous antibodies revealed conspicuous septa and polar bodies, apparently polysaccharide in nature.

After various degrees of lysozyme digestion of both living and heat-killed capsulated bacteria, the surface structures were demonstrated by the homologous antibodies (Tomcsik & Guex-Holzer, 1952), and it can be shown that at the appropriate stage of the reaction the cell wall and cross walls are rendered clearly visible to phase contrast by the same polysaccharide antibody which reacts with the septa and polar structures of the capsule.

This specific cell-wall reaction is not visible in intact cells, but can be shown in material in which the cytoplasm has been autolysed or digested with trypsin.

It may be argued that the specificity of this reaction is compromised by the use of digested bacteria; lysozyme will eventually destroy the cell wall completely (Welshimer & Robinow, 1949). Similarly, the final appearance of cells which have undergone this reaction might, because of the dilatation of the cell wall, be confused with the so-called 'capsular swelling reaction', which is discussed below. One purpose of this paper is, therefore, to indicate the occurrence of the specific cell-wall reaction under a wider variety of conditions, in both capsulated and non-capsulated bacteria.

MATERIALS

The strain *Bacillus* M, isolated and described by us (Tomcsik, 1950; Tomcsik & Guex-Holzer, 1951), was used in preference to typical strains of *B. megaterium* because of its stable production of mucoid colonies for 4 years on Gladstone & Fildes (1940) CCY agar, and because of the constant appearance in its capsule of the polypeptide-polysaccharide complex structure, even without added glucose in the medium. The non-capsulated form could regularly be obtained by culturing it in Gladstone & Fildes bouillon or Sauton medium, aerated by negative pressure in litre suction flasks, each containing 300 ml., with cotton-wool stoppered glass tubes extending below the surface of the medium.

D-Glutamic acid polypeptide antibody was produced in rabbits by ten to fourteen injections with suspensions of *B. anthracis*, grown on agar in 20% (v/v) CO_2, and killed by heating at 100° for 30 min.

The homologous serum, which did not contain polypeptide antibody, and will be designated the polysaccharide antibody, was prepared by immunizing two groups of rabbits with eight to ten doses of capsulated and of non-capsulated washed suspension of *Bacillus* M respectively, at intervals of 3 days.

METHODS

One drop of bacterial suspension was permitted to dry briefly on a cover slip, to cause the organisms to adhere, and a further drop of water was added to replace the evaporation. The cover slip was inverted on a slide and a loopful of undiluted serum was placed on the edge, so that it was drawn in by capillary action. By this means the majority of bacteria remained undisturbed by the currents created and it was possible to make serial photomicrographs of the same field before and after successive applications of sera. The capsular reaction was conducted, except where otherwise stated, with 24 hr. cultures of living bacteria; longer incubation or preliminary treatment, in the form of a 'microdissection' with trypsin or lysozyme or by autolysis, in order to destroy the cytoplasm, or at least to separate the cell wall from the cytoplasmic membrane, as described below, was necessary in order to demonstrate the specific cell-wall reaction. Photomicrographs were made as described by Tomcsik & Guex-Holzer (1953).

RESULTS

Untreated bacilli. The 24 hr. cultures showed mucoid colonies with capsulated bacilli of the 'rough' type (Bisset, 1948), usually with four cells but sometimes less or considerably more. Normally the capsule is invisible by phase contrast (Pl. 1, fig. 1), but when the polypeptide antibody is added a homogeneous capsule becomes visible in bacilli grown on agar; the bright, non-reactive zone between cell and capsule corresponds to the cell wall (Pl. 1, fig. 2). Whenever it proved possible to add polysaccharide antibody without disturbing the bacilli under the microscope which had already reacted with the polypeptide antibody, the following changes were observed (Pl. 1, fig. 3). Distinct septa appeared across the capsule, sometimes appearing to split into two layers on the convex side of curved chains; dark polar structures appeared, corresponding to the apparent depressions at the poles which could sometimes be observed with the polypeptide antibody alone; and in place of the previous homogeneous appearance of the capsule, a striated structure could be observed.

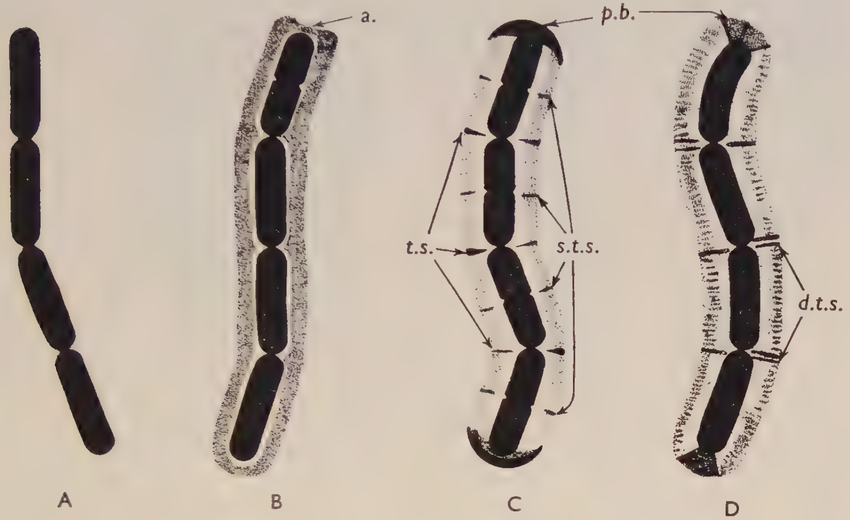

Fig. 1. A: *Bacillus* M by phase contrast without immune serum. B: the same bacillus after the addition of polypeptide antibody; *a* = polar depression. C: addition of homologous immune serum; *t.s.* = transverse septa; *s.t.s.* = secondary transverse septa (at the site of incomplete cell divisions); *p.b.* = polar body. D: the same as C in curved chain; *d.t.s.* = divided transverse septa.

These appearances were independent of the age of the culture, but a modified and very striking reaction was obtained if the growth from a 3 hr. aerated Sauton bouillon culture at 32° was centrifuged, and a small amount of sediment resuspended in saline. An initial marked motility was inhibited by homologous serum diluted up to 1:1200, indicating the presence of flagellar antibodies, but neither polypeptide nor polysaccharide antibody was capable of demonstrating any capsular material at this stage. If, however, the sediment was kept at room temperature, in the dark, for 3 days, the secondary growth showed loss of motility, with chain formation and very large capsules, which reacted to the antibodies with the production of very thick septa, extending across the chain and projecting beyond it at the edges (Pl. 1, fig. 4). On the convex sides of curved chains the double structure of these thick septa could clearly be seen (Pl. 1, fig. 6). The polar, polysaccharide structures were voluminous (Pl. 1, fig. 4), and, even with the polypeptide antibody alone, a striated appearance could be discerned (Pl. 1, figs. 5, 7), with gaps corresponding to the polysaccharide septa. It is only reasonable to assume that the entire striated structure of the polypeptide capsule is complementary to the striated polysaccharide described above. In Fig. 1 A–D we summarize schematically the most typical capsular reactions.

Autolysed bacilli. After incubation in CCY agar at 32° for some days, occasional bacilli showed disintegration of the cytoplasm, but only rarely of the capsule. Spore formation was not seen, but could be induced, in some individuals, on a medium containing potato extract. Pl. 2, figs. 8, 9, show such a culture before and after the addition of polysaccharide antibody. Several intact bacilli and a short chain with two spores can be seen. In the latter the cell wall is faintly visible by phase contrast, but the addition of the antibody demonstrates the capsules on the intact bacilli and shows up this cell wall as a thick, dark line. To obviate the possibility that this reaction was due to the presence of residual capsular material on the surface of the wall, a further experiment was made with non-capsulated bacilli grown for 24 hr. at 32° in aerated Sauton medium and permitted to autolyse under chloroform for 10 days at 37°. Their cytoplasm was fragmented and the cell wall was faintly discernible (Pl. 2, fig. 10), but gave a very distinct specific cell-wall reaction after the addition of polysaccharide antibody (Pl. 2, fig. 11). A constriction could be observed at the point of division.

Lysozyme-digested bacilli. The specific cell-wall reaction could be demonstrated on capsulated bacilli after moderate lysozyme digestion. The enzyme first loosens the capsular material, giving access to the cell wall, which then becomes detached from the cytoplasmic membrane, so that its reactions are clearly visible (Pl. 2, fig. 12). The essential stages of lysozyme action are illustrated in Fig. 2A–D, but the last stages are not shown. The naked cytoplasts may survive after dissolution of the wall; their contents go slowly into solution and finally the empty, spherical membrane also disappears. These changes occur in a similar manner when boiled bacilli are used, so that it appears improbable that autolytic enzymes play an important part in the major stages of the reaction. The coagulated cytoplasts, fixed in a rod shape, do not similarly dissolve, however, and at this stage autolytic enzymes may take part in the dissolution of normal bacilli.

Without the addition of the polysaccharide antibody, the cell walls of lysozyme-digested bacilli are only faintly visible (Pl. 2, fig. 13); but with antibody the cell wall and cross walls are clearly seen at an advanced stage of digestion. We consider that this observation makes it improbable that any simulation of a specific cell-wall reaction might be caused by the adherence to the wall of residual capsular substances.

Trypsin-digested bacilli. Trypsin digestion of both capsulated and non-capsulated bacilli was performed by adding 50 mg. (approx.) commercial trypsin (Siegfried, Zofingen) with chloroform to 10 ml. of saline suspension of bacteria, adjusted to pH 8 and incubated at 37° for 6–24 hr.

Under phase contrast, after addition of the polysaccharide antibody, the cell wall and capsule appeared almost intact; the latter somewhat shrunken (Pl. 3, fig. 16). The polar structures were very obvious; the cell wall appeared as a dark line, and the cross walls and capsular septa showed as a series of straight lines across the chain, alternatively thicker and thinner, along its length. This appearance strengthened our conviction that these capsular septa represented true cell divisions, of which the thinner examples were presumably the less mature. Digestion was most apparent within the cell wall, where all that remained was a small, sharply defined rod-like or spherical body, usually one per cell, and exhibiting marked Brownian movement. We cannot venture to speculate upon the identity of this body.

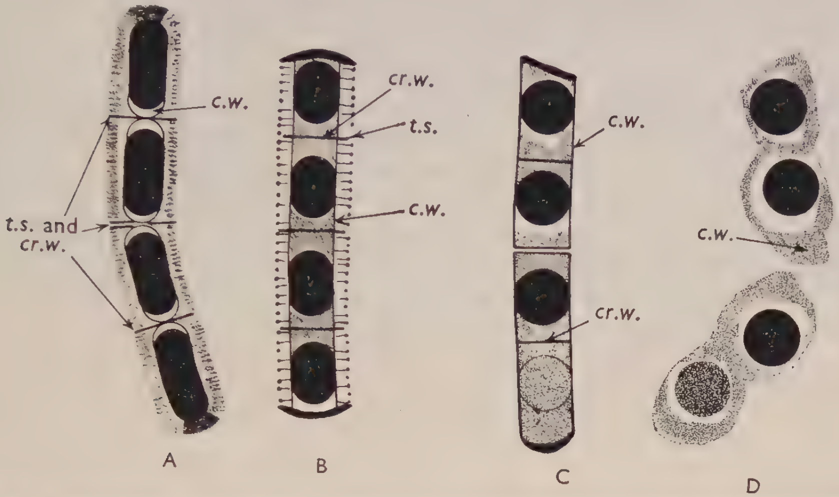

Fig. 2. Demonstration of the cell wall with homologous immune serum on lysozyme-treated bacilli. A–D = successive stages of lysozyme digestion; *c.w.* = cell wall; *cr.w.* = cross wall; *t.s.* = transverse septum. A: dilatation of the cell wall and initial stage of spherical transformation of the cytoplasm. A gradual disintegration of the capsule and the release of polypeptide and polysaccharide substances begins. B: the capsule is partially disintegrated; the cell wall and the cross wall also react with the polysaccharide antibody. The transverse capsular septa are thinner, but not yet totally disintegrated; they form a direct continuation of the cross walls. The spherical transformation continues in parallel with the further dilatation of the cell wall. C: the capsular structure, including the transverse septa, disappears entirely; the cell wall and the cross walls do not appear to be adversely affected and they react strongly with antibody. The cell wall at the end of the chains is thicker. The cross walls may show a double structure. The spherical transformation of the cytoplasm is complete; some of the spherical bodies may lose their original dark appearance. D: the chains are disarticulated. First the cross walls, then the cell walls disintegrate; they still react, however, with antibody before their final dissolution.

Shrinkage of the capsule after trypsin digestion was most apparent in the secondary growth forms of the originally non-capsulated bacilli from aerated Sauton medium (compare Pl. 1, fig. 4, Pl. 3, fig. 17), in which changes in the rigidity of the cell wall could also be seen (Pl. 3, fig. 17). These bacilli gave no cell-wall reaction with polypeptide antibody (Pl. 3, fig. 18), and no structure was revealed in the capsule by reaction at an inter-isoelectric pH with bovine plasma albumin (Tomcsik & Guex-Holzer, 1954), although a secondary swelling of the capsule occurred (Pl. 3, fig. 19). It was repeatedly observed that the residual globular bodies reacted at acid pH values; their size was considerably increased and their Brownian movement ceased entirely.

The non-capsulated bacteria reacted as was anticipated when digested with trypsin. Before the addition of serum the cell wall appeared as a thin line (Pl. 3, fig. 20). It reacted strongly with polysaccharide antibody and gave a non-specific reaction with proteins at acid pH values (Pl. 3, figs. 21, 22). The reaction of the small bodies with proteins at acid pH values was very marked (Pl. 3, fig. 22). The specific wall reaction, before and after the addition of polysaccharide antibody, was also observed in the same chain of trypsin-digested, non-capsulated bacilli (Pl. 3, figs. 23, 24).

DISCUSSION

These observations show that the bacterial cell wall reacts specifically with the appropriate antibody, and this specific cell-wall reaction can be rendered clearly visible to phase contrast by appropriate treatments.

The specific cell-wall reaction is elicited by a similar mechanism to that of the specific capsular reaction. The antibody reacts with the homologous antigen on the surface of these cell structures, and may penetrate within these structures to some extent, depending upon permeability. The capsular reaction, originally described by Neufeld (1902), is still designated (erroneously, in our opinion) a capsule swelling reaction. This is apparently due to a misreading of Neufeld's text. He did not at first envisage the existence of a capsule in his pneumococci and reported a swelling *of the bacterial cell*, as the capsule was rendered visible. In fact the main reaction is an antigen-antibody precipitation, causing changes in refractility; secondary capsular swelling occurs only occasionally in certain types of bacteria, and the non-specific capsular reaction, depending upon protein precipitation within the capsular substance at inter-isoelectric pH (Tomcsik & Guex-Holzer, 1953, 1954) leads more frequently to shrinkage.

If the principle underlying both specific cell-wall and capsular reaction is the same, and if a particular antibody reacts equally well with the cell wall and with the transverse septa of the capsule, then it appears that the same substances must take part in the formation of both. Our previous studies (Tomcsik & Guex-Holzer, 1951, 1952) indicate that this particular antibody reacts with polysaccharides, but it remains to be proved, by the absorption of the serum with purified bacterial extracts, that a single substance is concerned. We hope to be able to report soon upon our immunochemical studies on the identity of this material in the cell wall and capsular septa, but the present work eliminates the possibility that D-glutamic acid takes part in these structures.

Apart from these similarities, the close relationship between the capsular septa and the cellular cross walls is indicated by our morphological observations. The latter structures designated 'cross walls' (Knaysi, 1951) or 'transverse cell walls' (Bisset, 1948, 1950) do not stain well with basic dyes, and the term septum is more frequently used to describe a transverse growth of the basophilic cytoplasmic membrane, preceding division. By our methods we could not distinguish septum and wall in this sense, and although our capsular structures had no affinity for dyes, we use the term septum because of its more general connotations.

The question of whether complex structures can be produced by and upon the cell wall, we now consider to have been answered in the affirmative. But whether the cytoplasmic membrane takes part in this production, and what is the relationship of these polysaccharide structures to the cell wall, we do not yet know.

This investigation was aided by a grant from the Rockefeller Foundation. Our thanks are due to Dr K. A. Bisset for revising the text and to Miss A. Beckmann for technical assistance.

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EXPLANATION OF PLATES

Bacillus M by phase-contrast microscopy. All plates at ×2500.

Plate 1

Fig. 1. Capsulated bacteria; no antibody.

Fig. 2. Capsulated bacteria; polypeptide antibody.

Fig. 3. Capsulated bacteria; first polypeptide, afterwards polysaccharide antibody (the same microscopic field as in fig. 2).

Fig. 4. Originally non-capsulated bacteria from aerated Sauton culture; secondary capsule production in the centrifuged sediment at room temperature in 3 days. Polysaccharide antibody. Note the exceptionally thick transverse septa.

Fig. 5. Bacteria as in fig. 4. Polypeptide antibody. The distribution of the secondarily produced polypeptide is not homogeneous. Gaps in place of the polysaccharide transverse septa.

Fig. 6. Bacteria as in fig. 4. Polysaccharide antibody. Swollen bacteria. In a few places, at the convex side of the curved chains, split transverse capsular septa.

Fig. 7. Bacteria as in fig. 4. Polypeptide antibody. Exceptionally inhomogeneous distribution of polypeptide material. Larger gaps in place of the polysaccharide transverse capsular septa.

PLATE 2

Fig. 8. Four-day old potato-agar culture. In the majority capsulated bacteria. No antibody. Spores in one chain.

Fig. 9. The same microscopic field as in fig. 8. Polysaccharide antibody. Specific cell-wall reaction on the sporulating chain and specific capsular reaction on the other bacteria.

Fig. 10. Non-capsulated bacteria from aerated Sauton culture, autolysed. No antibody; the cell wall is barely visible.

Fig. 11. The same bacterium as in fig. 10. Polysaccharide antibody. The cell wall appears as a thick line.

Fig. 12. Capsulated bacteria digested with 1:100 egg white for 10 min. at room temperature. Polysaccharide antibody. Cell-wall and cross-wall reaction. Capsular structure partially detached. The remnants of capsular transverse septa are located at the prolongation of the cross walls. The spherical transformation of the cytoplasm is complete.

Fig. 13. Non-capsulated bacteria from aerated CCY (Gladstone & Fildes) bouillon, partially digested with lysozyme. No antibody. The cell wall is barely visible. The spherical transformation of the cytoplasm is not yet complete.

Fig. 14. Bacteria prepared as in fig. 13. Polysaccharide antibody. Cell-wall and cross-wall reaction. Spherical transformation of the cytoplasm is further advanced.

Fig. 15. Similar preparation to fig. 14. The specific cell-wall reaction is pronounced. In one place unusual spherical deformation of the cell wall.

PLATE 3

Fig. 16. Capsulated bacteria digested with trypsin. Polysaccharide antibody. Specific cell-wall and capsular reaction. Note the trypsin-resistant cytoplasmic bodies.

Fig. 17. Similar preparation to fig. 16. Polysaccharide antibody. The trypsin digestion is more pronounced.

Fig. 18. The same bacterial suspension as in fig. 17. Polypeptide antibody. No cell-wall reaction, no capsular structure.

Fig. 19. Originally non-capsulated bacteria from aerated Sauton culture; secondary capsule production in the centrifuged sediment at room temperature in 3 days. Trypsin digestion. No antibody. Non-specific capsular reaction with bovine plasma albumin Fr. V; buffer pH 2·6. Enlargement and immobilization of the cytoplasmic bodies, not digested with trypsin.

Fig. 20. Non-capsulated bacteria from aerated Sauton bouillon, digested with trypsin. No antibody. The cell wall can be discerned as a very thin line.

Fig. 21. Bacteria as in fig. 20. Polysaccharide antibody. Specific cell-wall reaction.

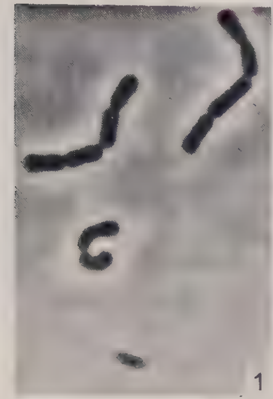
Fig. 22. Bacteria as in fig. 20. No antibody. Non-specific cell-wall reaction with bovine plasma albumin Fr. V; buffer pH 2. Enlargement and immobilization of the trypsin-resistant cytoplasmic bodies.

Fig. 23. Bacteria as in fig. 20. No antibody. The cell wall can hardly be discerned.

Fig. 24. The same field as in fig. 23. Polysaccharide antibody. Very clear specific cell-wall reaction.

(*Received 5 October* 1953)

Journal of General Microbiology, Vol. 10, *No.* 2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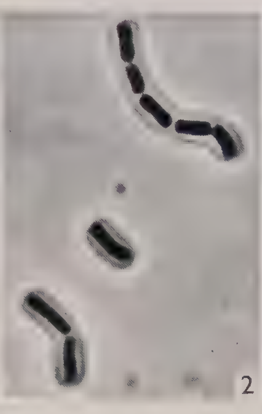
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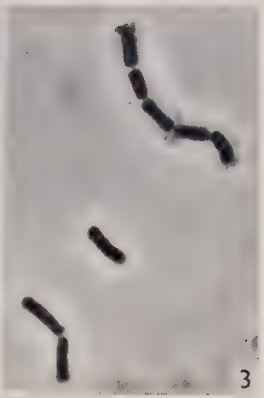
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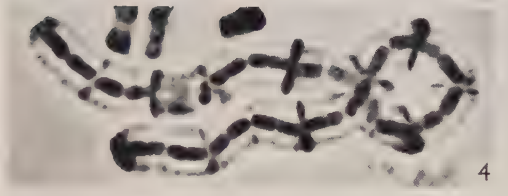
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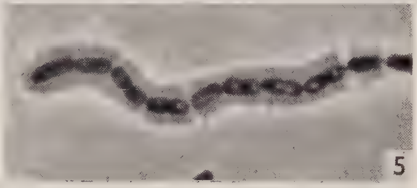
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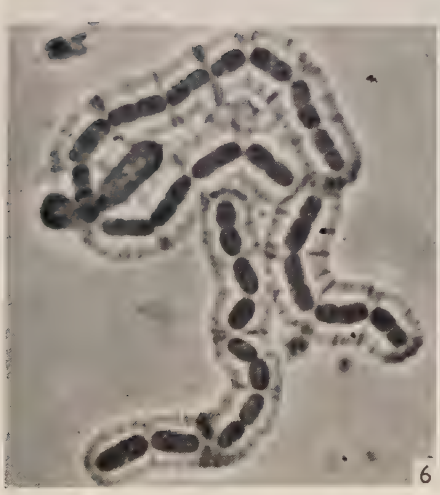
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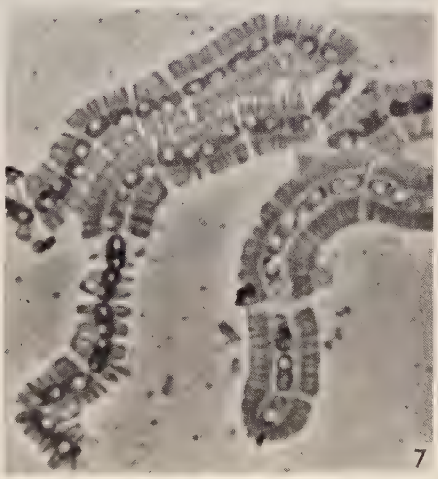
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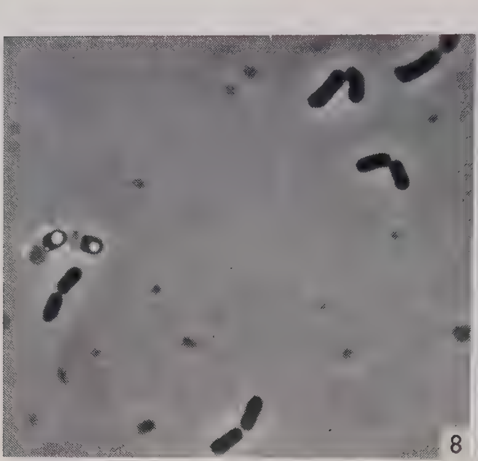
J. Tomcsik & S. Guex-Holzer—A cell-wall reaction. Plate 1



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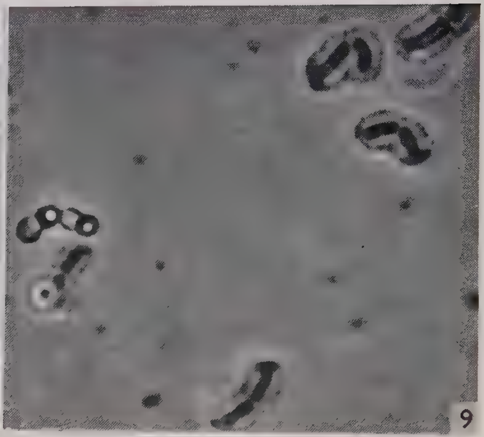

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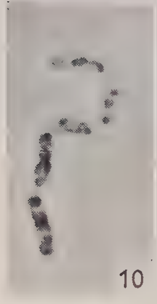

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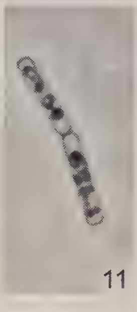

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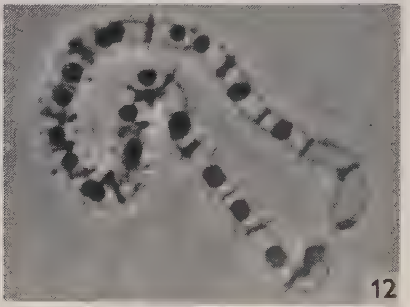

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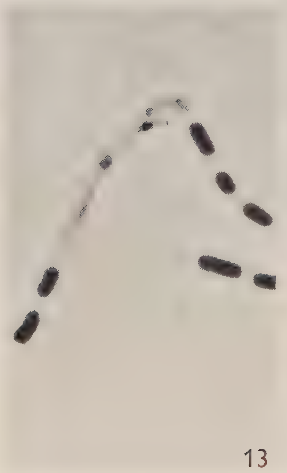

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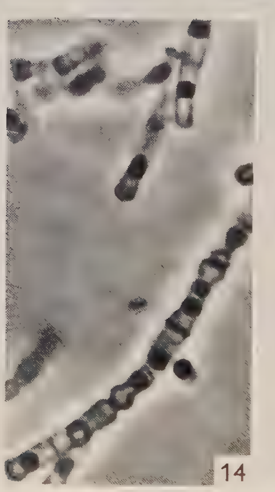

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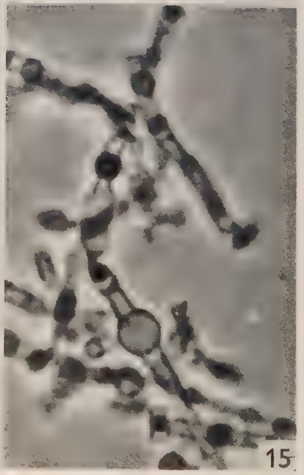

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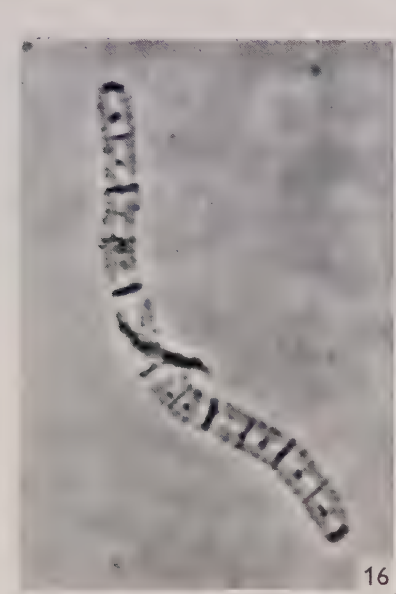
J. Tomcsik & S. Guex-Holzer—A cell-wall reaction. Plate 2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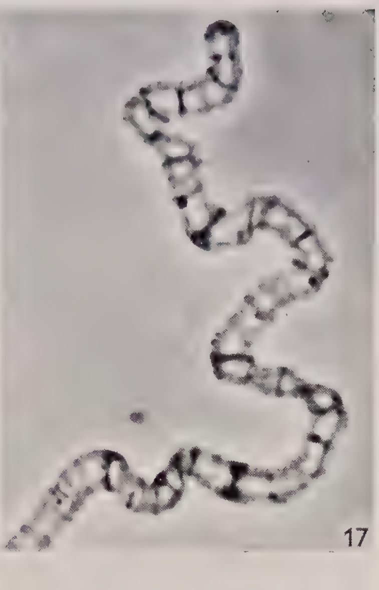
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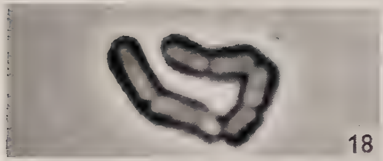
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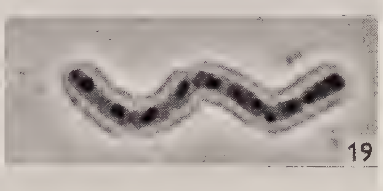
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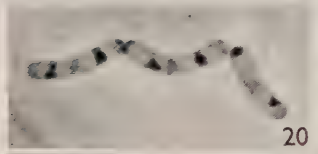
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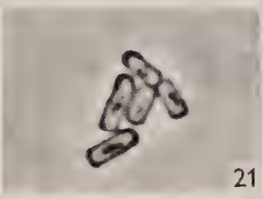
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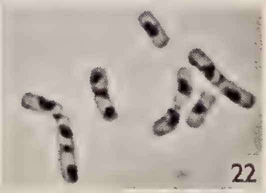
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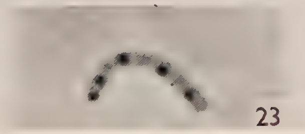
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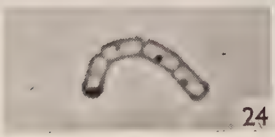
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J. Tomcsik & S. Guex-Holzer—A cell-wall reaction. Plate 3

GRACE, J. B. (1954). *J. gen. Microbiol.* **10**, 325–327

Some Observations on the Flagella and Blepharoplasts of *Spirillum* and *Vibrio* spp.

BY JOYCE B. GRACE

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SUMMARY: The flagella of certain large spirilla appear to be compound structures composed of a large number of individual fibres. These arise in bundles from a single blepharoplast, whereas in other bacteria each flagellum arises separately. The flagella of spirilla are thus, in some respects, intermediate between those of other bacteria and of flagellates.

Since the description of a 'thread-like trunk' on cells of *Ophidomonas* (*Thiospirillum*) spp. which behaved as an organ of propulsion (Ehrenberg, 1838) numerous observations have been made on the flagella of both living and stained cells of large spirilla (Bütschli, 1902; Ellis, 1902; Fuhrmann, 1909; Reichert, 1909; Buder, 1915; Yuasa, 1936; Pijper, 1949). These have confirmed that most spirilla possess a large number of very fine flagellar strands, but have not adequately differentiated these complex structures from the multiple, lophotrichous flagella of, for example, *Pseudomonas* spp. Several workers (Bütschli, 1902; Fuhrmann, 1909; Yuasa, 1936) suggested that the flagella arose from a single basal structure analogous to the blepharoplast of the flagellates.

More recent studies with the electron microscope (van Iterson, 1947, 1953; Lofgren, 1948; Houwink, 1953) have shown that the origin of the flagella is intracellular but have not elucidated completely their mode of attachment. Van Iterson (1953) failed to find any evidence of a blepharoplast after autolysis of the cells, whereas Lofgren (1948) described a disk-like structure to which the flagellar strands were attached. Houwink (1953) observed a small hook at the base of each flagellar strand similar to those on the flagella of other bacteria. Blepharoplasts have, however, been observed in other bacterial groups, notably *Proteus* (Houwink & van Iterson, 1950), *Vibrio* (van Iterson, 1953), *Pseudomonas* and *Bacterium* spp. (Bisset & Hale, 1951). In these it was shown that each flagellum arose from a separate granule.

From their structure, as seen by the electron microscope, and from their behaviour, the flagella of large spirilla appear to be compound structures consisting of numerous slender fibrils which arise from a single blepharoplast and function as a single organ in a manner analogous to that of the simple, but much stouter flagellum of, for example, *Vibrio* spp. They are, thus, distinct from the multiple, separate flagella of other bacteria.

METHODS

To discover the general arrangement of their flagella, cells of *Spirillum volutans*, *Sp. undula*, *Thiospira* sp. and *Rhodospirillum rubrum* were examined and compared with *Vibrio cholerae*. The cells were suspended in distilled water, dried upon collodion or 'formvar' films, shadowed with metallic gold and examined with the electron microscope.

By this method, however, the mode of attachment of the flagella was not visible and a strain of *Rhodospirillum rubrum* was selected for further study. Cells from a 24 hr. culture were suspended in distilled water and incubated at 5° for 18 hr. After this time, a large proportion of the cells appeared to be empty when stained with methylene blue. The bulk of the cells was then centrifuged off, and the supernatant was examined by the electron microscope.

OBSERVATIONS

Most of the spirilla examined possessed from ten to thirty or more flagellar fibres at one or both ends of the cell. These were usually arranged in one or more bundles making the total number difficult to count (Pl. 1, figs. 1, 2). Even when the flagellar fibrils were separate from one another, closer scrutiny showed that apparently single strands were often composed of more than one unit. The average diameter of each unit, as measured from electronmicrographs, is approximately 10 $m\mu$., whereas that of the average flagellum of *Vibrio* and *Pseudomonas* spp. lies between 20 and 28 $m\mu$.

In the electronmicrographs of the untreated cells, the flagella, usually from one to four in number, appeared to arise in bundles, but the cell contents were too opaque to the electron beam to show their mode of attachment (Pl. 1, fig. 1).

The strain of *Rhodospirillum rubrum* selected for further study possessed comparatively few flagella. After 18 hr. at 5° a large proportion of the cells appeared to be partially or completely empty when viewed with the electron microscope. The flagella, however, were still attached to the cells and in many they appeared to arise from a single basal granule (Pl. 1, figs. 4–6).

In contrast, the cells of *Vibrio cholerae* which were examined possessed single, stout polar flagella. In several electron micrographs it was observed that the flagellum arose in the cell from a spherical structure about 100 $m\mu$. in diameter similar to the basal granules seen in other groups of bacteria.

DISCUSSION

In the bacteria in which blepharoplasts have previously been demonstrated, and also as shown here in *Vibrio cholerae*, each flagellum appears to be attached to a separate basal granule or blepharoplast, whereas in spirilla the much smaller flagellar fibrils arise in bundles from the same blepharoplast, and constitute a single, complex organ.

This type of structure is intermediate between the typical bacterial flagellum and those of other types of Protista.

I wish to thank Miss C. M. F. Hale and Mr C. C. Newton for their assistance in making these electron micrographs.

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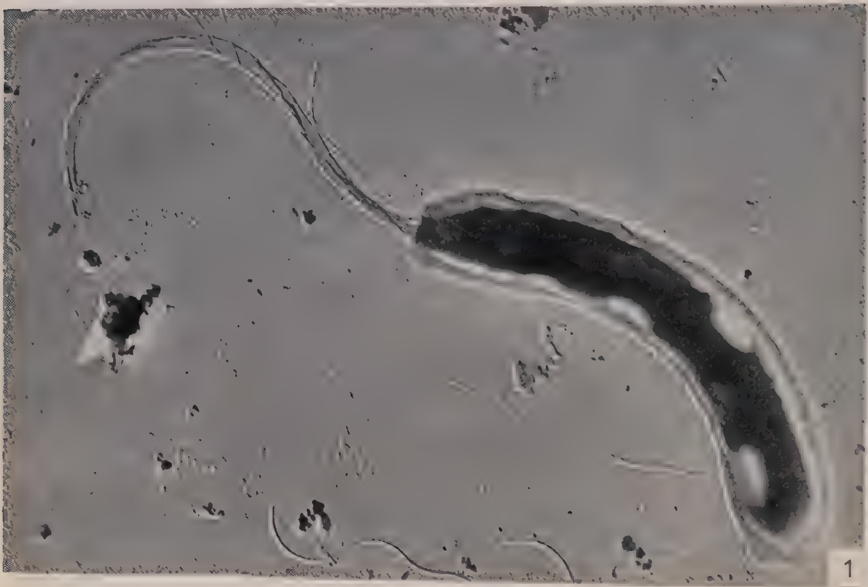
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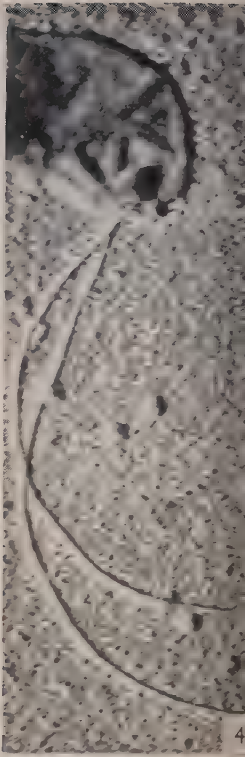
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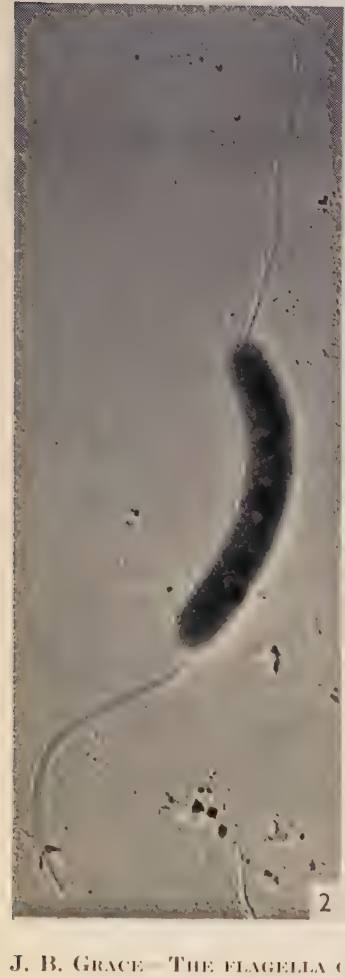
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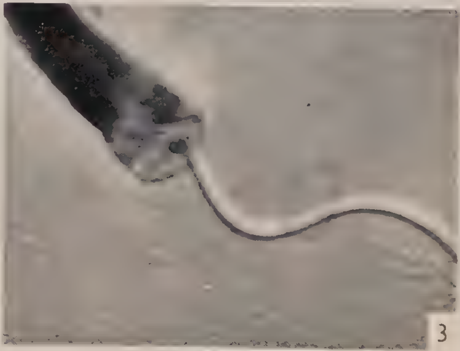
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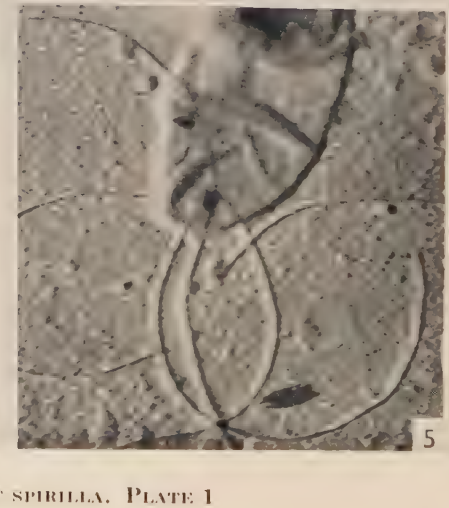
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J. B. GRACE—THE FLAGELLA OF SPIRILLA. PLATE 1

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EXPLANATION OF PLATE

Fig. 1. *Thiospira* sp. showing three bundles of fibrils emerging from the cell and becoming united into a single strand. Electron micrograph, gold shadowed, ×10,000.

Fig. 2. *Thiospira* sp. showing flagellar fibrils arranged in a single bundle. A similar appearance to that observed in living cells. Electron micrograph, gold shadowed, ×5000.

Fig. 3. *Vibrio cholerae* showing a single, thick flagellum attached inside the cell to a basal granule. Electron micrograph, gold shadowed, ×25,000.

Figs. 4–6. *Rhodospirillum rubrum* autolysed cells showing the flagellar fibrils attached to a single basal structure. Electron micrographs, gold shadowed, ×25,000.

(*Received* 9 *October* 1953)

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Some Observations on the Physiology of *Saccharomyces rouxii* Boutroux

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SUMMARY: *Saccharomyces rouxii* is shown to be tolerant of a wide range of acidities and of sugar and salt concentrations, but sensitive to temperatures above 35° unless protected by a small quantity of sugar in the medium.

A limited investigation into the vitamin nutrition and nitrogen requirements of the yeast is reported.

The work described in this paper forms part of an investigation into the fermentation of malt extract by the osmophilic yeast *Saccharomyces rouxii* Boutroux (English, 1953), and was undertaken in order to throw further light on factors affecting that fermentation.

Little physiological work has been carried out with this yeast, either under its present name, or that under which it was originally identified for me by Dr Kreger van Rij (English, 1953) *Zygosaccharomyces japonicus* Saito, though Fabian & Quinet (1928) and Fabian & Hall (1933) found its thermal death-points in various substrates to the nearest 5°.

Among other osmophilic yeasts, an extensive investigation into the metabolism of *Z. acidifaciens* has been carried out by Nickerson & Carroll (1945), but they were mainly interested in factors affecting its respiration, and in the products of the fermentation. Nickerson & Thimann (1943) studied some aspects of the vitamin requirements of the same yeast. Kroemer & Krumbholz (1931, 1932) and Krumbholz (1931*a*, *b*) have made detailed studies of the effect of temperature and concentration of substrate on various osmophilic yeasts from wine must.

MATERIALS AND METHODS

The isolate G1 of the malt extract strain of *S. rouxii*, described previously (English 1953), was used for all experiments, though in some cases other isolates were used in addition, and gave exactly similar results. Stock cultures were maintained as before, and the same basal medium was used, to which enough glucose was added to give the required osmophilic conditions. Glucose was chosen for these experiments in preference to maltose (the predominant sugar in malt extract) because, being a monosaccharide, there is no danger of further hydrolysis and because the osmotic pressure of a given solution is greater than that of a maltose solution of equal concentration. The solubility of L-glucose in water at 25° is about 45% (w/w): solutions as concentrated as 69% (w/w) can, however, be obtained by slight initial warming of the syrup, as mutarotation of some of the L-glucose to the more soluble β-glucose occurs (Jackson & Silsbee, 1922). Though in a metastable condition, these solutions were found to be quite satisfactory in practice, crystallization occurring seldom, and then only in the 69 % solution.

For work on the pH range of the yeast, the pH of the sterile medium was adjusted aseptically, in bulk, and the medium was then tubed aseptically. Any two consecutive compounds of the following four—H_3PO_4 (1·0 N); KH_2PO_4 (1·0 M); K_2HPO_4 (1·0 M); and NaOH (1·0 N)—were used as buffers, to a total of 10 ml. in 250 ml. medium. As it had previously been observed that the pH of the uninoculated media drifted somewhat during storage at 30°, all adjusted media were stored at this temperature for 3 days before use, by which time a steady value was reached.

To obtain spores for thermal death-point determinations the yeast was at first grown on slopes made from commercial plaster of Paris, moistened with distilled water, but the percentage viability of spores obtained under these conditions was low. Subsequently it was found that spores with a high percentage viability were formed very freely on 2-week old malt agar slopes, and these spores were used for the determinations.

All treatments were carried out in duplicate, and experiments were repeated at least once. Points on graphs represent average results for one experiment. Incubation was at 30° unless otherwise stated.

Growth of the yeast in liquid cultures was measured on a Hilger and Watts 'Spekker' absorptiometer, results being recorded as readings from the drum of the instrument without attempting to estimate the number of cells actually present. Owing to the increasing transparency of yeast cells in media of increasing concentration, comparison of growth of the yeast at different sugar concentrations by this method is not valid. Differences in behaviour at the same concentration, brought about by varying other enviromental conditions, may, however, be compared.

Serial readings of pH were made colorimetrically with B.D.H. capillator tubes, a convenient method where only very small samples are available and great accuracy is not essential.

Thermal death-points were determined by the capillary tube method.

RESULTS

Adaptability to media of different concentrations

Saito (1907), in his original isolation of *Z. japonicus* from 'koji', used a 17 % solution of sodium chloride, with suitable nutrients, as a selective medium; hence it is of interest to compare the reaction of the malt extract isolate in this and other concentrated media. The yeast was therefore grown in the basal medium to which different concentrations of salts and sugars were added, with the results shown below.

Glucose. Fermentation of a 69 % (w/w) solution is apparent in 5 days, the cells appearing normal in size and shape.

Maltose solution containing 90 g. sugar in 100 ml. water shows visible fermentation in 5 days.

KNO_3. Very sparse growth occurs in a saturated solution.

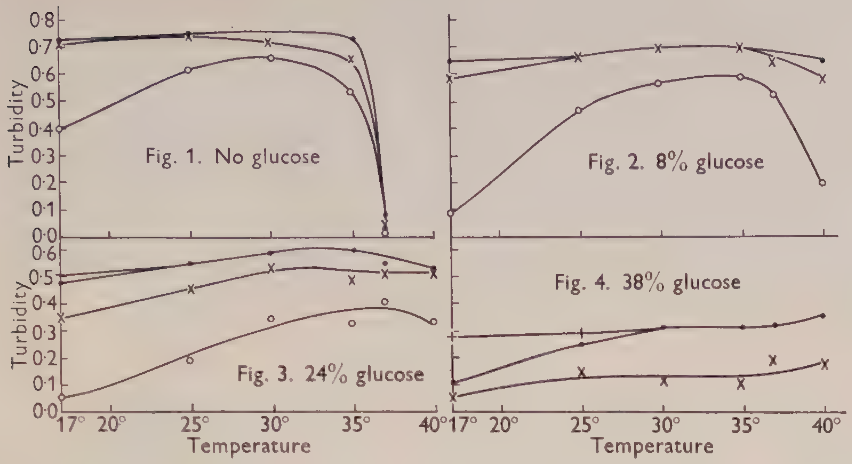
NaCl. The yeast can grow sparsely in a 17 % (w/v) solution, but the cells are malformed and grow in clumped colonies, apparently due to the failure of buds to separate completely from the parent cells.

Saito does not mention malformed cells in his cultures in 17 % salt solution, so it seems probable that the malt extract strain of the organism differs from the 'koji' strain in this respect. The rapid and luxuriant growth of the yeast in the basal medium alone, shows that it is a facultative rather than an obligate osmophile. This is also borne out by the observation that after prolonged cultivation on basal medium alone, the yeast loses its power to ferment 69 % glucose solutions, but can be 'retrained' by successive transfers through media of increasing sugar content, a phenomenon also reported for osmophilic yeasts by Ingram (1950) and Scarr (1951).

The adaptive capacity of *S. rouxii* when transferred quickly from highly concentrated media to very dilute ones, and vice versa, is remarkable. Cultures grown in 61 % (w/w) glucose solution can be transferred to distilled water (with immersion in one intermediate dilution lasting about 15 min. necessary to reduce the density of the medium sufficiently to allow sedimentation of the cells on centrifuging) with apparently no effect on their viability; even after soaking in water for 24 hr, viability is only slightly decreased. The same holds true when cultures grown in the basal medium are transferred to 61 % glucose solution. Microscopic observations revealed no obvious damage to the cells.

Effect of temperature and concentration of the medium

In this experiment the yeast was grown in liquid media of varying glucose concentrations, which were incubated at different temperatures. The cultures were sampled aseptically on successive days and growth was measured on the 'Spekker' absorptiometer. The experiment was carried out on the basal medium alone, and with the addition of 8, 24 and 38 % (w/w) glucose at temperatures of 17, 25, 30, 37 and 40°. Turbidity readings were taken until no further increase was recorded. The results are shown graphically in Figs. 1–4. These figures show that the presence of glucose in the medium, even in as low a concentration as 8 %, gives the yeast complete protection against temperatures otherwise lethal to it; without glucose it can grow slightly at 37° and not at all at 40°, while with 8 % glucose suboptimum or optimum growth can take place at the higher temperature. The time taken for the yeast to die when stored at 40° in basal medium alone was determined, in order to discover whether this temperature merely inhibited or actually killed the organism in the absence of sugars. A vigorous 3-day old culture in malt broth was stored at 40°, and subcultured at intervals on to malt agar incubated at 30°. The yeast was found to be alive, but with greatly impaired viability, after 80 hr., and dead after 96 hr. These results may be compared with the effect of incubation at 37° on *S. rouxii* growing in malt extract, where the still more concentrated medium allows growth for 4 weeks, but the yeast succumbs eventually (English, 1953).



Figs. 1–4. The effect of temperature on *S. rouxii* grown in glucose solutions of different concentrations. Fig. 1. Basal broth without additional glucose. Fig. 2. Basal broth with 8 % glucose. Fig. 3. Basal broth with 24 % glucose. Fig. 4. Basal broth with 38 % glucose. ○ = second day of incubation; × = fourth day; ● = seventh day; + = ninth day.

Thermal death-points

These were determined for vegetative cells and spores in distilled water and in 70 % (w/v) maltose solution, as any substance more viscous could not be handled in capillary tubes. Vegetative cells were obtained from 48 hr. cultures on basal medium, scraped off and suspended in the test liquid: sporing cultures were taken from similar slopes 14 days old in which the presence of a large percentage of spores was confirmed microscopically. The results after 5, 10 and 15 min. exposure are shown in Table 1.

Thermal death-points for honey and maple syrup strains of *Z. japonicus* have been determined to the nearest 5° by Fabian & Quinet (1928), and

Table 1. *Thermal death-points of vegetative cells and spores of* Saccharomyces rouxii

	Distilled water							
	Vegetative cells				Spores			
Time (min.)	46°	47°	48°	49°	55°	56°	57°	58°
5	++	++	++	–	++	++	+	–
10	++	+	+	–	++	+	–	–
15	+	–	–	–	++	–	–	–

	70 % maltose							
	Vegetative cells				Spores			
Time (min.)	48°	49°	50°	51°	58°	59°	60°	61°
5	++	++	+	–	++	++	+	–
10	++	–	–	–	++	+	–	–
15	++	–	–	–	+	–	–	–

++ = normal growth, + = poor growth, – = no growth.

Fabian & Hall (1933), and to the nearest 1° for *S. rouxii* by Boutroux (1884), who, however, did not state whether spores or vegetative cells were used.

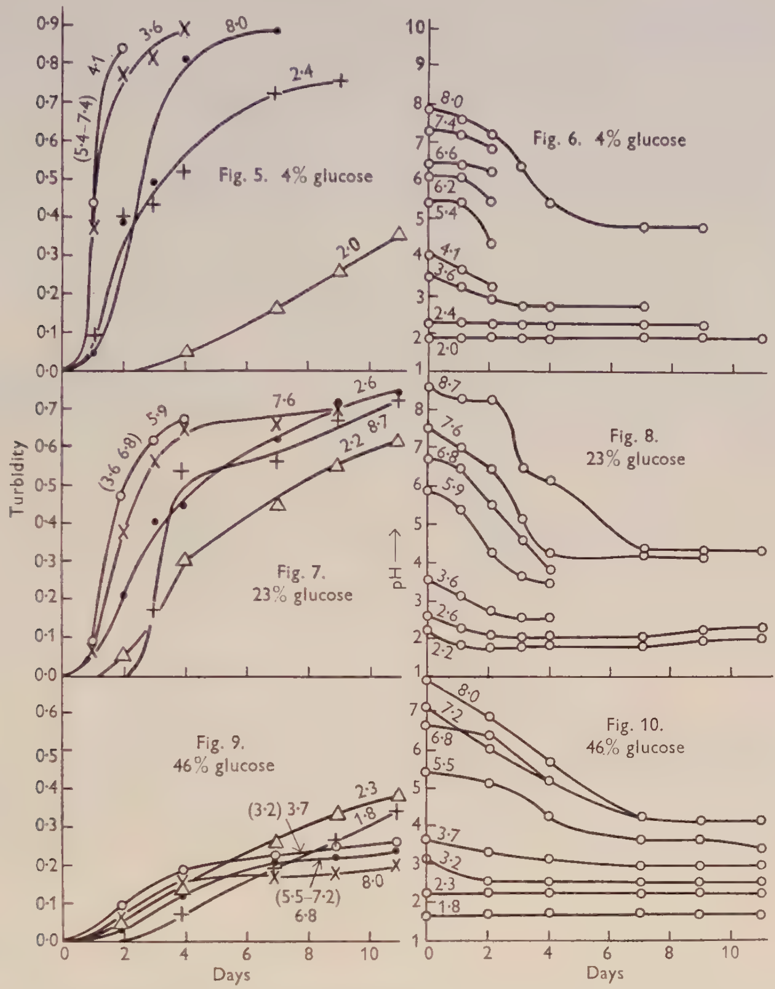
Though these investigators obtained their thermal death-points by different methods, so that absolute comparison of their results is impossible, all agree that there is an approximate difference of 10° or more between the temperatures required to kill vegetative cells and spores. This contrasts with most of the results of Lund (1951) who found differences of 2–4° between spores and vegetative cells of most of his strains of *S. ellipsoideus*, *S. turbidus* and *S. odessa*. Only in one strain of *S. odessa* was there a difference of 12°.

Effect of pH *on growth*

This was studied in the basal medium with three levels of glucose, 4, 23 and 46 % (w/w). A range of pH values in each medium was obtained as has been described, and the growth of the yeast and acidity of the medium were determined on samples withdrawn aseptically at intervals throughout the experiment. The results obtained are shown in Figs. 5–10.

In all media, one further turbidity reading was made than is indicated by the last point on the appropriate graph. In 4 and 23 % glucose (Figs. 5, 7) these readings were below those made on the preceding sample. Jordan & Jacobs (1948) noticed a similar effect when the food content of the medium in which they were growing *Escherichia coli* reached starvation level. In 46 % glucose (Fig. 9), on the other hand, they remained approximately the same or rose very slightly, indicating that growth had not quite ceased. It will be noticed that in the two less concentrated media, total growth was apparently submaximal at those pH values that allowed of the fastest growth rate (Figs. 5, 7). Although analogous observations have been made by others, in this case it seems that the result can be accounted for by the conditions under which the experiment was carried out. For the only times at which it was possible to take turbidity readings were those actually shown on the curves. These were adequate for the slow-growing cultures, but where conditions were very favourable and growth was rapid, the interval between two readings at the end of the growth period was sufficient to allow turbidity to reach its peak and then fall off, so that a true maximum was never recorded.

The figures show that the malt extract isolate of *S. rouxii* can tolerate an extremely wide pH range, from 2·0 to 8·7, and thus may differ considerably from Boutroux's original strain, of which he said 'Elle résiste mal l'acidité.' If, however, the medium in the alkaline range could be buffered against acid produced by the metabolism of the yeast, it is doubtful whether these pH levels would be so well tolerated by the malt yeast. For an examination of the curve for pH 8·7 in Fig. 8 shows that the organism only grows at a pH above 8 for a comparatively short time, during which the growth rate as shown by the corresponding curve in Fig. 7 is slow. It is only when the yeast, by its own metabolism, has acidified the medium, that the growth rate increases. The relative humidity of a 46 % glucose solution being about 87 %, this result may be compared with that obtained by Scarr (1951) for her osmophilic yeast strain 21, which failed to grow in 54 % (w/w) sucrose solution (relative humidity over 90 %) at pH 7·8.



Figs. 5–10. The effect of the pH of the medium on *S. rouxii* grown in glucose solutions of different concentrations. Figs. 5, 7 and 9. Growth of the yeast in media containing 4, 23 and 46 % glucose respectively. Figs. 6, 8 and 10. Change in pH of media containing 4, 23 and 46 % glucose respectively, due to the growth of *S. rouxii*. The initial pH of the medium is marked on each curve. Figures in brackets indicate that media of these initial pH values gave results almost identical to the curve over which the figures appear.

Nutrition

A little preliminary work has been carried out on the vitamin nutrition and nitrogen requirements of *S. rouxii*, of which a short report follows. All glassware for these experiments was cleaned in chromic acid, and culture tubes were covered with metal caps instead of cotton-wool plugs. The basal defined medium contained 4 % glucose, 0·2 % KH_2PO_4, 0·1 % $MgSO_4 . 5H_2O$, 0·025 %

$CaCl_2$, and 1 ml. lactic acid per 100 ml. medium, and was adjusted to pH 5·0 with KOH.

Vitamins. 0·4 % $(NH_4)_2HPO_4$ was added to the basal defined medium, and the following vitamins were tested alone and in combination (per 100 ml. medium): biotin, 0·2 μg., inositol 1000 μg., calcium D-pantothenate 100 μg.

The results appear in Table 2 and show that biotin, which is known to occur in malt extract, is the only one of these vitamins essential for growth.

Table 2. *Vitamins necessary for the growth of* Saccharomyces rouxii

Vitamin	Growth	Vitamin	Growth
None	–	Biotin + inositol	+ +
Biotin	+ +	Biotin + Ca pantothenate	+ +
Inositol	–	Inositol + Ca pantothenate	–
Ca pantothenate	–	Complete	+ +

+ + = growth and fermentation. – = no growth.

Table 3. *Growth of* Saccharomyces rouxii *on various nitrogen-containing compounds*

Heavy	Moderate	Poor	None
Casein hydrolysate	Adenine	Arginine	Choline
	Alanine	Valine	Cystine
	Asparagine		Hydroxyproline
	Glutamic acid		Isoleucine
	Glutamine		
	Guanine		
	Tryptophan		
	Uracil		
	Xanthine		

Lochhead & Landerkin (1942), in their study of the vitamin requirements of osmophilic yeasts, divided their organisms into three groups according to whether pantothenic acid was unnecessary, stimulatory or essential for their growth. In this scheme, my isolate of *S. rouxii* falls into the first group, in that neither pantothenic acid nor inositol is essential or stimulatory. Thus the malt yeast differs in its vitamin requirements from the strain of *Z. japonicus* isolated from normal honey, on which these authors worked, as pantothenic acid was essential to their organism.

Nitrogen. For these experiments 0·2 μg. biotin/100 ml. was added to the basal defined medium, and a number of amino acids were tested as possible sources of nitrogen, at a concentration of 1 mg./100 ml. Negative and positive controls were included, the latter containing 0·4 % $(NH_4)_2HPO_4$. The results are shown in Table 3.

DISCUSSION

These experiments make it clear that *S. rouxii* is a facultative rather than an obligate osmophile, and that it is an adaptable organism, tolerant of a wide range of environmental conditions, its power of survival at temperatures over 35° being greatly enhanced by the addition of a small quantity of sugar to the medium.

Of those who have worked previously on yeasts identified as *Z. japonicus*, only Fabian & Quinet mention the lower limits of sugar concentration at which the yeast can grow, and then only indirectly, in that *Z. japonicus* is included in the list of species isolated from honey, and they state that they were unable to obtain any osmophilic yeasts from honey in media containing 10% or less of sugar. If the latter observation applies to any sample containing *Z. japonicus*, their yeast behaved in a very different manner from the malt organism, but from the data it is impossible to be sure of this. It is known that other osmophilic yeasts can grow in very dilute media; for instance, Ingram (1949) was able to grow an unnamed yeast from concentrated orange juice, on potato glucose agar.

The temperature relationships of *S. rouxii* differ from those reported for other osmophilic yeasts, where these have been investigated, in the striking effect of comparatively low concentrations of sugars in protecting the organism against otherwise lethal temperatures. Krumbholz (1931*a*) found that 30% (w/v) of sugar was insufficient to allow growth of four strains of *Z. polymorphus* at 40°, but that good growth was obtained with 40% sugar. In a fifth strain, feeble growth occurred at 40° in 30% (w/v) sugar, but none in a 20% syrup. *S. rouxii*, however, can grow at this temperature in the presence of as little as 10% (w/v) (8%, w/w) glucose, when without it is killed after 96 hr. exposure.

It is difficult to imagine that so small a quantity of sugar can bring about such an effect only by the slight increase in osmotic strength of the solution, yet it must apparently cause some radical change in the metabolism of the cells or in their equilibrium with the environment.

I should like to thank the following for their assistance: Mr J. T. Gunner and Miss Constance Rainbow of this Department for invaluable technical help: Dr C. J. Hickman and Dr C. Rainbow of the University of Birmingham for reading and criticizing the manuscript: Dr S. W. F. Underhill of this Department for his interest in and encouragement of the work; and the Directors of The British Drug Houses Ltd. for permission to publish the results of the investigation.

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(*Received* 2 *November* 1953)

WILLIAMS, R. E. O. (1954). *J. gen. Microbiol.* **10**, 337–341

Glucuronidase Production by Serotypes of *Streptococcus pyogenes*

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SUMMARY: Seven hundred and thirty-six strains of *Streptococcus pyogenes* were examined for production of glucuronidase. The types most commonly found producing the enzyme were 9 and 28. Among other types there appeared to be some inverse relation between production of glucuronidase and of the M antigen.

Two recent reports (Robinson, Blinn & Frank, 1952; Jacox, 1953) have shown that the ability to produce glucuronidase varies among the different serological types of *Streptococcus pyogenes* (Lancefield's group A). In both studies the type identification of the streptococci was based exclusively on the M precipitin method of Swift, Wilson & Lancefield (1943) and a large proportion of the strains (22 and 39 %) were untypable. Moreover, a substantial proportion of the untypable strains produced glucuronidase. In this laboratory, Griffith's (1934) slide-agglutination method of typing is used as well as Lancefield's precipitin method, and we are thus able to identify, to some extent, most of the strains that are untypable by the precipitin method (Williams & Maxted, 1953). It therefore seemed worth investigating the glucuronidase production of a number of strains that had been typed by our routine.

MATERIAL AND METHODS

This report is based on a total of 736 strains isolated by constituent laboratories of the Public Health Laboratory Service, and by a number of laboratories in Northern Ireland, during 2 weeks, 17–23 November 1952 and 10–16 May 1953, in a survey carried out to determine the distribution of types then current in the country. The strains were isolated from patients and healthy carriers. All were shown to belong to group A.

Type identification was carried out here or in the Regional Laboratories of the Service at Cambridge, Cardiff and Oxford. All strains were tested by slide agglutination and subsequently by precipitation. Strains that were not agglutinated by any sera, and a few that gave very complex agglutination patterns were tested in the precipitin test against all the type sera available; otherwise the precipitin test was used to confirm or make more precise the type diagnosis based on the agglutination test.

Phenolphthalein glucuronide was prepared by my colleague Mr T. Nash from a sample of the cinchonidine base made by Mr B. W. L. Brooksbank (Barber, Brooksbank & Kuper, 1951) and kindly given me by Dr Mary Barber. It was incorporated at a concentration of 0·0002 M in serum agar. The streptococci under test were grown overnight in 10 % (v/v) serum broth at 37° and a loopful was spotted on to the surface of plates of the glucuronide agar. These were incubated overnight at 37° and then exposed to ammonia, as in the method of Barber & Kuper (1951). Strains were considered to have produced glucuronidase when a distinct pink colour developed around the colony. Preliminary tests with the strain of *Str. pyogenes* Blackmore (type 11) as used by Robinson *et al.* showed that under these conditions the glucuronide concentration used was optimum. With 0·5 % (w/v) glucose in the glucuronide agar none of twenty glucuronidase-producing strains produced any detectable breakdown of the glucuronide in this test (cf. Jacox, 1953).

RESULTS

In Table 1 are set out the types of the strains, determined both by agglutination and precipitation methods, and their glucuronidase production. The order is arranged to bring together those types, such as 3 and 13, which are known to share antigens. In some cases the agglutination test gave a complex pattern and, when the precipitin reaction was negative, the type could not be determined; it was, however, often possible to tell that the strain was probably related to one or more of the recognized types. Such strains are entered under headings such as 'Misc. 3, 13, B 3264'.

Of the 736 strains tested, seventy-nine (10·7 %) produced glucuronidase; this was only common among strains of two types, namely type 28 and type 9, although eight of the ten strains that were agglutinated by the serum for type 12 but failed to give a precipitin reaction were also positive. It should be noted that of the types giving unclassifiable agglutination patterns, nine were agglutinated by sera for type 9 and also for type 18 or 30, and of these seven produced glucuronidase; this may indicate that they should really be regarded as type 9.

Of the 736 strains, 353 gave a reaction in the M precipitin test [and thirty-seven precipitated with the serum for type 28, in which the type-specific antigen is different from the typical M antigen (Lancefield & Perlman, 1952)]. Of these 353 strains 4·3 % produced glucuronidase. This compares with 11·8 % of the 346 strains that produced no demonstrable M antigen, and 62·6 % of the thirty-seven type 28 strains.

It has been our experience that anti-M sera for precipitin typing are very much easier to prepare and more satisfactory to use with some types than with others (Williams & Maxted, 1953). The satisfactory types, of those represented in Table 1, are 1, 2, 3, 24, 29, 5, 12, 6, 14, 19, 30 and 18. There was a total of 384 strains distributed over these twelve types, of which 324 (85 %) gave a precipitin reaction and sixty failed to precipitate. Only three (0·9 %) of the 324 precipitin-positive strains produced glucuronidase, compared with nine (15·0 %) of the sixty precipitin-negative strains (difference = 14·1 %, standard error = 2·4).

For some other types, represented in the present series by 4, 9, 22 and 25, we have found the preparation of satisfactory anti-M sera to be difficult, and with the sera available precipitin reactions are commonly negative (86 % in the present collection). Of the 202 strains of these types, 13·4 % produced glucuronidase, but although none of the four strains of type 4 that produced

Table 1. *Glucuronidase production by serotypes of* Streptococcus pyogenes

	Type reaction in		No. of strains tested	Strains producing glucuronidase	
'Type'	Precipitin test	Agglutination test*		No.	%
1	1	1; –†	88	0	
	–	1	4	1	(25·0)‡
2	2	2	14	1	7·1
	–	2	7	0	
3	3	3±13, B 3264; –	61	2	3·3
	–	3	29	0	
13	–	13	9	3	(33·3)
	–	Misc. 3, 13, B 3264	19	1	5·3
4	4	4	14	0	
	–	4	91	4	4·4
24	24	–	3	0	
29	29	4	2	0	
28	28	28±4 (11/27/44§)	37	23	62·2
5	5	5±11, 12, 27, 44; –	49	0	
11	–	11	2	0	
12	12	12±5, 11, 27, 44; –	68	0	
	–	12	10	8	80·0
	–	Misc. 5, 11, 12, 27, 44 including some reacting also with 3, 13	53	5	9·4
6	6	6; –	14	0	
	–	6	3	0	
8/25	25	25±8	4	2	(50·0)
	–	25; 8/25; Imp. 19	23	4	17·4
9	9	9, 9/18	11	10	90·9
	–	9	36	5	13·9
14	14	14	5	0	
	–	14	3	0	
18	18	18; 9/18; 18/30; –	6	0	
	–	18	1	0	
19	19	±15, 17, 19, 23, 47; –	12	0	
30	30	±15, 17, 19, 23, 47; –	2	0	
	–	30	1	0	
	–	Misc. 18, 22, 23, 30	1	0	
22	–	22	23	2	8·7
Unclassifiable	–	9/18	7	6	(85·7)
	–	9/30	2	1	(50·0)
	–	1/13/B 3264; 4/22; 4/27; 14/Imp. 19; 18/27/44	13	1	7·7
Negative			9	0	
Total			736	79	10·7

* Under 'agglutination test' are given the type numbers of the sera that agglutinated the strains. The entry 3±13, B 3264, etc., indicates that some of the strains were agglutinated by type 3 alone, some by 3 and 13, some by 3, 13 and B 3264 etc. Entries such as 9/18 indicate definite agglutination patterns.

† – = no reaction.

‡ = percentages in parentheses are based on less than ten strains.

§ = one strain.

glucuronidase gave precipitation with the M antiserum, ten of the fifteen type 9 and two of the six type 25 strains did so.

Summarizing these results in a different way (Table 2), of the seventy-nine strains which hydrolysed the glucuronide, only 3·8 % gave M precipitin reactions with sera known to be satisfactory, and 51·9 % gave no precipitin reaction at all. No less than 29·1 % of the remainder were type 28.

Table 2. *Typing reactions of* 79 *glucuronidase-producing strains of* Streptococcus pyogenes

	No. of strains	% of total	
Typable by precipitation			
with 'satisfactory' anti-M sera	3	3·8	48·1
with anti-M sera of doubtful value	12	15·2	
with anti-R serum for type 28	23	29·1	
Typable by agglutination only			
in types usually giving good M precipitinogens	9	11·4	51·9
in types rarely giving good M precipitinogens	15	19·0	
in types for which no anti-M sera were available (11 and 13)	3	3·8	
but giving only pattern reactions	6	7·6	
Agglutination reactions complex and unclassifiable	8	10·1	
	79	100·0	

DISCUSSION

The results reported here in part confirm, but also extend, the earlier observations of Robinson *et al.* and of Jacox. The overall percentage of glucuronidase-producing strains, 10·7 %, lies between the 16·1 % reported by Jacox, and the 4·0 % of Robinson *et al.*; the frequency of the enzyme production by type 28 also confirms Jacox's findings. Jacox reported that 'all the strains of types 11, 13, 25 and 28...contained glucuronidase', but this was based on single strains of types 11, 13 and 25. In the present series three of nine type 13, six of twenty-seven type 8/25, but neither of two type 11 strains, were active. Type 9, which we found active, was not recognized as a glucuronidase-producing strain by the previous workers, doubtless because of the difficulty of recognizing this type by the precipitin method. It is further of some interest that even among types readily recognized by the precipitin test, the strains which failed to give a precipitin reaction were significantly more often able to hydrolyse glucuronide than those which gave a typical precipitin reaction.

The previous workers observed that glucuronidase production was common among strains untypable by the M precipitin method. From the present work it is clear that this derives partly from the ability of some types not recognizable by the precipitin test to produce glucuronidase. It is also partly due to an apparent inverse relation between ability to produce glucuronidase and M antigen demonstrable within particular types. Lastly, the only type in which glucuronidase production is really common and which is readily recognizable by the precipitin test is type 28, and in this type the antigen recognized in the standard precipitin test differs from typical M antigens in several ways.

My thanks are due to Mr P. Jerram for technical assistance.

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(*Received* 6 *November* 1953)

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The Nuclear Structures of *Oscillospira guilliermondi* Chatton and Perard

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SUMMARY: Cytological studies of *Oscillospira guilliermondi* from the caecal contents of guinea-pigs show that this organism consists of trichomes with numerous disk-like cells containing nuclei in the form of paired rods. They offer a peculiarly clear illustration of this typically eubacterial form of nucleus.

Oscillospira guilliermondi is a relatively large multicellular micro-organism first described by Chatton & Perard (1913) in the caecal contents of the guinea-pig. These workers were unable to demonstrate any nuclear structures, and for this reason they considered it to be a member of the Myxophyceae, with which classification many subsequent workers have concurred. Baker (1933, 1943) found *O. guilliermondi* in ox rumen and in guinea-pig caecum, and was similarly unable to show nuclear structures of any kind, although Simons (1922), working with guinea-pig material, described a series of nuclear changes preceding spore formation which Delaporte (1934) confirmed. Moir & Masson (1952) described a similar organism from the rumen of sheep, and were of the opinion that it was divided into a number of segments, each of which contained one nucleus. Although a nucleus is now accepted as being present in this interesting organism, little real detail concerning its structure has so far been produced; the observations presented here suggest that it is eubacterial rather than algal in character.

Robinow (1942) used the acid-Giemsa technique for the first convincing demonstration of bacterial nuclear structures, which he and subsequent workers have shown, in the vegetative stages, to consist of paired rod-like bodies (Robinow, 1945; Bisset, 1950). By the use of this method, an exceedingly clear demonstration of a nucleus of this type in *O. guilliermondi* can be achieved.

MATERIALS AND METHODS

As *O. guilliermondi* has yet to be grown in pure culture, its cytology was studied in preparations made from guinea-pig caecal contents in which it occurs very frequently. Thin films were made on cover-slips and stained without fixation, by the acid-Giemsa technique as described by Bisset (1950). Hydrolysis in N-HCl for 13 min., and staining in dilute Giemsa for 3–4 min., gave very good results.

OBSERVATIONS

Mature individuals of *O. guilliermondi* measured 5–7μ. in width, and from 20 to 70μ. in length, and consisted of a trichome of narrow disk-like cells. Each cell contained a nucleus in the form of two rod-like bodies, parallel to each other and at right-angles to the long axis of the trichome. The paired nature of the nucleus is seen particularly well in Pl. 1, figs. 1, 2. The nuclei in any one trichome did not all lie in the same plane, and the paired rods presented all aspects from profile to end-on. The individual organism in Pl. 1, fig. 1, shows the nuclear rods of five adjacent cells lying in planes which are alternately at right-angles. As may be expected of rod-like structures, those seen in profile appear less intensely light-absorbent than those seen from the end, since a greater depth of stained material is presented in the latter case. This interpretation of their form appears more probable, therefore, than the alternatives that they represent rods or disks of varying length or diameter, as their intensity would then be either indifferent or greater in the larger (profile) examples, rather than in the smaller (end-on) examples.

It is not clear from the brief description of Moir & Masson (1952) exactly what they consider to constitute a single cell. Presumably an entire individual is implied, whereas it is obvious from the photomicrographs shown here that each organism consists of from ten to thirty or more cells. The transverse septa, stained by Giemsa, are clearly seen between each nucleus in Pl. 1, figs. 1, 3 and 4. Fully formed cross walls, appearing as unstained gaps in the length of the organism, can be seen in Pl. 1, fig. 2.

Most authors are agreed that *O. guilliermondi* forms endospores in a manner typical of Eubacteria. Delaporte (1934) described the full cycle of events in the process, which she claimed to be comparable with that occurring in spore-forming bacteria. Her account is based on iron-haemotoxylin stained preparations which are less clear and less easily interpreted than is the acid-Giemsa stained material presented here. Even from these, a complete understanding of the process cannot yet be obtained, but it appears that the nuclei at points *a*, *b* and *c* (Pl. 1, figs. 3, 4) are in various stages of condensation preliminary to spore maturation. It is probable that *a* represents the most, and *c* the least, advanced stages.

DISCUSSION

Chatton & Perard (1913), Simons (1922), and Baker (1943) accepted *O. guilliermondi* as a member of the Myxophyceae, whereas Fritsch (1945) doubts the validity of such a grouping on the grounds that its mode of spore formation is not typical of the class. Moir & Masson (1952), following Peshkoff in the 6th edition of *Bergey's Manual* (1948), placed it with the true bacteria; so also did Pringsheim (1949), who stated that Robinow had demonstrated the possession of peritrichous flagella by *O. guilliermondi*. To this bacterial character is now added that of the possession of typically bacterial nuclei in the form of paired transverse rods. *O. guilliermondi* thus affords a very good example of a typical bacterial nucleus, and because of its size permits this structure to be more accurately observed than in smaller genera, and shows that the vegetative nucleus consists of paired rods, which can be observed in various aspects, and not of disks as has been occasionally suggested. It also shows the advanced degree of multicellular structure which can be observed in some micro-organisms. Although considerably larger, *O. guilliermondi* closely resembles *Caryophanon* spp. both in morphology and habitat, and may reasonably be presumed to belong also to the Order Eubacteriales, as defined by Bisset & Moore (1950).

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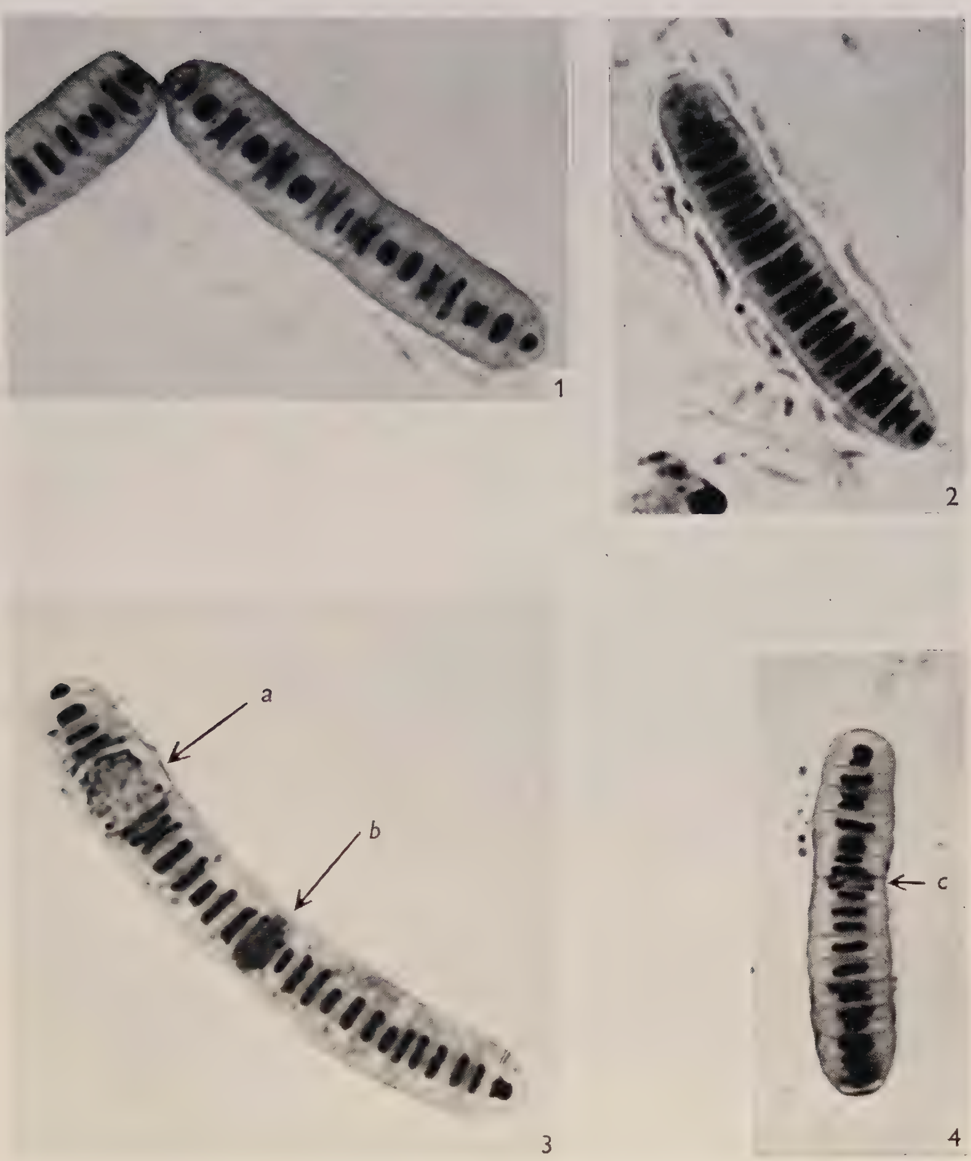
EXPLANATION OF PLATE

Fig. 1. *Oscillospira guilliermondi*; each cell contains one nucleus, consisting of a pair of rod-like bodies. Acid-Giemsa, × 1000.

Fig. 2. *O. guilliermondi*; nuclei. Mature cross walls can be seen as unstained gaps in the length of the trichome. Acid-Giemsa, × 1000.

Figs. 3, 4. *O. guilliermondi*; nuclei. Nuclear condensation preliminary to spore formation can be seen at points *a*, *b* and *c*. Acid-Giemsa. × 1000.

(*Received* 10 *November* 1953)



A. A. TUFFERY—NUCLEAR STRUCTURES OF *O. GUILLIERMONDI*. PLATE 1

No. 1, *Volume* 10 *of the Journal of General Microbiology was issued on* 12 *February* 1954.
No. 2, *Volume* 10 *of the Journal of General Microbiology was issued on* 9 *April* 1954.

NOSSAL, G. J. V. & DE BURGH, P. M. (1954). *J. gen. Microbiol.* **10**, 345–352.

Ectromelia Virus Multiplication in Mouse Liver

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SUMMARY: The multiplication of ectromelia virus in mouse liver cells has been studied using intravenous inocula of 10^6 to 10^{10} LD50 doses. About 30% of this virus is found in the liver in 5 min., and most of this disappears as infective virus in the next hour. After a lag period, the length of which depends on the number of LD50 doses/cell which initiated the infection, the virus titre increases again to the 5 min. level. Subsequently, steps of multiplication, each separated by a lag period, occur within each cell. The efficiency of virus multiplication over the range tested is independent of the infecting dose.

Since the classical experiments of Ellis & Delbrück (1939), who demonstrated that bacterial virus multiplication occurred in stages, it has been found that many animal viruses follow similar patterns. Briefly, these stages are: attachment of virus on to host cell; disappearance of part or all of infectivity; a phase during which no new infective virus can be demonstrated; a period of intracellular rise in infective titre followed by release of this virus which may infect further cells and repeat the cycle. Such growth curves are conveniently studied in the fertile hen's egg, and findings similar to the above description have been made using influenza virus (Henle, Henle & Rosenberg, 1947), vaccinia (Briody & Stannard, 1951), Newcastle Disease virus (Gordon, Birkeland & Dodd, 1952), meningopneumonitis virus (Girardi, Allen & Sigel, 1952), and other viruses. The study of multiplication of animal viruses in their natural hosts is made difficult by many factors which tend to obscure the events in any one cell. Some of the important factors are: (*a*) the difficulty of determining how many cells are infected by the inoculum; (*b*) the heterogeneous cell populations of the organs most commonly studied (lung and brain); (*c*) the infective titre which at any time represents the net result of virus formation and removal, by lymph or blood, or destruction, or entry into further susceptible cells. Fenner (1949) pointed out that the multiplication of ectromelia virus in the mouse was an excellent model for the study of host-virus relationships.

The following studies were undertaken in an attempt to lessen some of the above difficulties and thus to demonstrate a one-step growth curve of ectromelia virus in mouse liver. A preliminary account of this work has been published (Nossal & de Burgh, 1953).

MATERIALS AND METHODS

Mice. Albino Swiss mice, 6–10 weeks old, were used for the growth-curve experiments; similar mice or heterogenetic coloured mice of the Walter and Eliza Hall Institute (Melbourne) strain were used for the bio-assays of infectivity.

Virus. The 'Moscow' strain of ectromelia virus was used throughout. Stock suspensions of virus were made by grinding infected livers in a mortar previously chilled to −15°, and homogenizing with nutrient broth, the ice crystals formed acting as an abrasive. These suspensions were stored in sterile test tubes plugged with cotton-wool at −15°; they retained their titre unchanged for over 2 months. When stocks of very high titre were required they were prepared as follows. Mice were inoculated intravenously with large doses of virus, and were sacrificed late in the infection, but before they became moribund; the livers were removed and homogenized in 10 % sucrose solution at 0–4°, in a pestle and mortar, to make a 10–15 % suspension of liver. Then the suspension was subjected to either of the following procedures to concentrate the virus. (1) The suspension was centrifuged 1 to 3 times at 4000 *g* for 8–12 min. to remove cell debris, red blood cells, nuclei and mitochondria, and the supernatant fluid centrifuged at 23,000 *g* for 12–20 min. to deposit the virus. Sometimes the suspensions were layered over 30 % sucrose solution to achieve better separation. (2) The suspension was centrifuged once at 1500 *g* for 10 min. and the supernatant centrifuged at 23,000 *g* for 12 min. to give a pellet which contained most of the virus.

Method (1) has the virtue of yielding suspensions of virus much freer from contaminating cell debris, but it requires more manipulations, and as no refrigerated centrifuge was available, the preparations got rather warm with a resultant decrease in titre. Method (2) results in a bulky pellet which also contains most of the mitochondria. This can be homogenized in a chilled (−15°) mortar and rendered sufficiently disperse to allow the intravenous injection of 0·1 ml. By either method, the final suspension represents the virus from up to 2 g. liver in 1 ml.

Growth curves. These were studied by one or both of two techniques:

(1) Large numbers of mice were inoculated intravenously at times known within 5 min. for each mouse. With very concentrated stock suspensions, a proportion of mice died immediately. Mice were taken in groups of 3, 4 or 5 at intervals and killed by decapitation. The livers or large samples thereof were removed, inspected, weighed and ground with nutrient broth in the manner described, stored at −15°, and titrated within 2 weeks.

(2) Parts of the growth cycle can conveniently be studied in one mouse. The mouse is infected and anaesthetized just before material is taken to obtain the first point on the curve. Of many anaesthetics tried, the most satisfactory was urethane (about 0·15 ml. of 25 % (w/v) solution/20 g. mouse, given intraperitoneally). This is quick to act (1 min.), not lethal in effective doses, and wears off very slowly (> 12 hr.). At no stage was there any evidence that the infective process was altered in any way by the anaesthetic. An incision $\frac{3}{4}$–1 in. long was made in the abdominal wall in the mid-line, and a lobe of the liver brought through the wound to the surface. Then either a small piece (*c.* 20 mg.) was cut from the edge of the liver and bleeding immediately stopped by application of a small piece of gelfoam (Upjohn and Co.) soaked in a solution of thrombin (200 i.u./ml.) or a whole lobe of liver was removed. The latter gives more satisfactory control of bleeding and larger pieces of liver are obtained.

By these methods, two to six samples can be obtained from one mouse, covering a period as long as 8 hr.

Histology. All mice were given 0·5 ml. 20 % (w/v) glucose in water by intraperitoneal injection at 90 and 45 min. before killing, to ensure that all cells capable of synthesizing and storing glycogen had enough substrate to do so. Small pieces of liver were fixed in Bouin-Allen or 80 % ethanol, and paraffin sections (8 μ.) cut. They were stained with haematoxylin and eosin or with the periodate-Schiff stain for glycogen.

Cell counting. The number of glycogen-deficient cells in twenty-two fields at a magnification of 168 were counted. This represented the total number of deficient cells in 22 × 450, i.e. 9900 liver cells, there being approximately 450 cells/field. This was taken as the number of glycogen-deficient cells/10,000. When a relatively large proportion of liver cells was deficient, fewer cells were counted, but never less than ten fields.

Direct measurements of the number of liver cells were made after the method of Brues, Drury & Brues (1936).

Virus titration. The livers were ground in chilled mortars with nutrient broth as described above, and bioassays of infectivity were made, using the Reed & Muench (1938) method of calculating the mouse LD 50. Serial twofold (0·3 $\log_{10}$) steps were used, with three mice per step; the standard error was calculated by the Pizzi (1950) formula, and was usually about 0·2 log unit. Occasionally, preliminary titrations using a modification of the Ipsen (1944) method were used.

RESULTS

It was noted by de Burgh (1950) that one of the first demonstrable changes in liver cells infected with ectromelia virus was loss of ability to store glycogen. This phenomenon can be used to study the extent and spread of infection in the liver. When a count is made of the fraction of liver cells which show glycogen deficiency, when this change is well established (18 hr. or more after infection), and this number is multiplied by $1·4 \times 10^8$ (the number of liver cells was found to be $1·4 \times 10^8$/g. liver with a standard deviation of $0·25 \times 10^8$), Table 1 can be constructed.

Table 1. *Relationship between the amount of virus injected and the number of cells unable to form glycogen*

$\log_{10}$ LD 50 doses injected	$\log_{10}$ no. of liver cells glycogen deficient	No. of deficient cells/100 LD 50 doses injected
6·1	5·85	60
6·5	6·25	56
6·8	6·45	45
7·1	6·85	56
7·5	7·25	56
7·8	7·45	45
8·1	7·65	35
9·0	8·10	12
9·2	8·10	8
10·0	8·15	1·4

Further, when we inject an inoculum and measure the infectivity of the liver 5 min. later, we obtain the relationship shown in Table 2. From Table 2 it appears clear that the proportion of virus in an infecting dose which is adsorbed to the liver in 5 min. is fairly constant over a large range of values, and is about one-third.

Table 2. *Proportion of injected virus found in the liver 5 min. after injection*

Log LD 50 doses injected	Log 5 min. titre	Percentage of inoculum found in liver at 5 min.
6·0	5·8	63
7·9	7·4	31
8·0	7·6	40
9·2	8·8	40
10·0	9·3	20

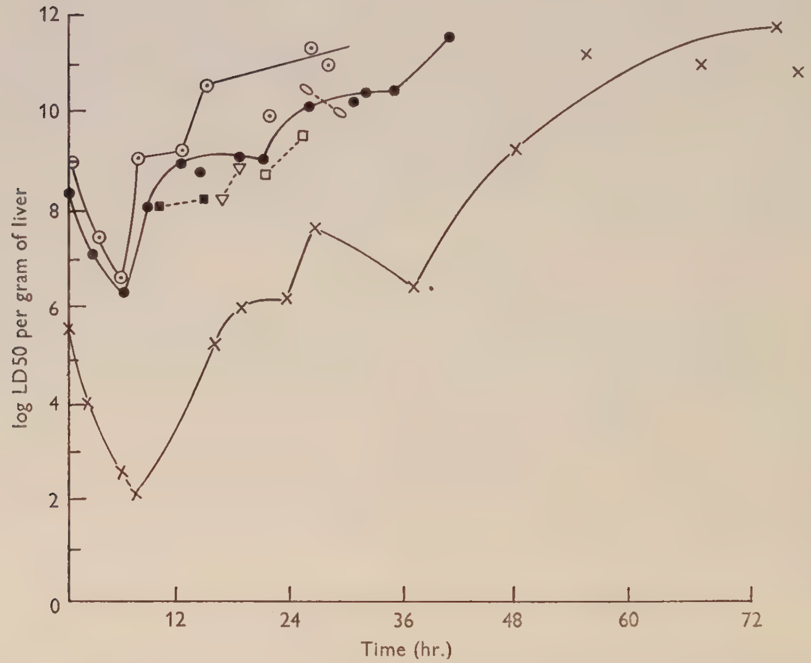

Fig. 1. Virus growth curves. The solid lines are virus growth curves at three different levels and each point represents a pool of (usually) three mice. Each pair of points joined by a dotted line represents an individual mouse.

From Table 1, it can be seen that with inocula over a range of 10^6–10^8 LD 50, the ratio of the number of deficient cells to the inoculum varies from about one to three to one to two, so in this range two LD 50 produce two to three glycogen-deficient cells. With inocula of 10^9 LD 50 and over, practically all liver cells are infected at zero time, as judged by glycogen deficiency, and Table 2 shows that the average infection per cell is more than one LD 50.

Fig. 1 shows the result of three experiments demonstrating the growth cycles of ectromelia virus multiplication in the liver, using seed inocula of 10^{10}, 10^9 and 10^6 LD50. In the experiment with the highest dose, 0·2 ml. of virus concentrate proved immediately fatal. We therefore gave two injections of 0·1 ml. separated by 5 min., and took the first titration point 7·5 min. after the first injection. At this time, some of the first injection was already disappearing. An addition of 0·3 log unit to the titre obtained would therefore seem to be a truer indication of the amount of virus reaching the liver. An initial liver titre of $10^{9\cdot3}$ LD50 represents 14 LD50/cell, and this figure is used

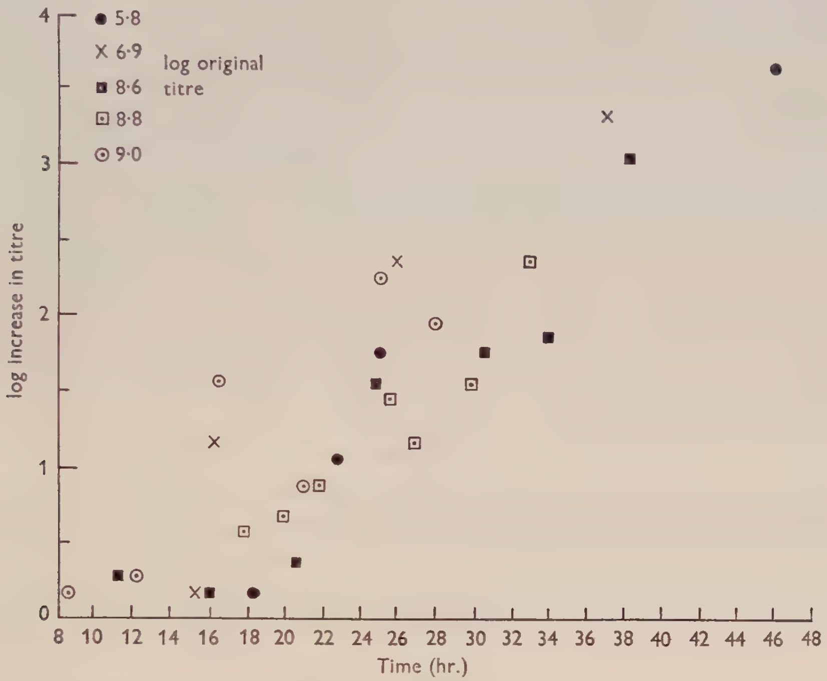

Fig. 2. Rate of increase of virus. The logarithm of the increase in titre, i.e. log titre at various times minus log 5 min. titre, is plotted against time in hours, for various dose levels.

in later discussion, although the titre actually found, giving 7 LD50/cell, appears in Fig. 1. Fig. 2 is a compendium of all our results over a large range of inocula, and plots the amount of virus multiplication that has taken place against time.

The growth curve in Fig. 1 obtained with an inoculum of 10^6 LD50 seems at first sight to show features very similar to those demonstrated in other systems, namely attachment of virus to liver, maximum at 3 min., disappearance of most of this during the next 8 hr., and then stepwise increase in infectivity separated by lag periods, becoming exponential after the third rise. A consideration of the other curves, however, shows that the explanation is not so simple. In these, practically all the cells are infected at zero time, i.e. there is practically no population of fresh susceptible cells to be infected by the first release of newly formed virus. Yet the increase in virus continues and is still stepwise. We must therefore conclude that the increase of virus titre in any one cell is stepwise.

On closer study these further facts become apparent:

(*a*) With a multiplicity of infection of 2·8 LD 50/cell, the first rise in infectivity occurs earlier than with less than 1 LD 50/cell, and with 14 LD 50/cell, it occurs earlier still.

(*b*) The titre after the first rise is quite close to the 5 min. value, and this was true in all our experiments. At the end of this rise, there is one LD 50 for every one present at 5 min.

(*c*) After this first rise, the titre remains constant for a variable period of time, depending on the initial dose, and then an increase of some 20 to 40-fold takes place.

(*d*) This rise is followed by another lag period. In the weakest dose curve there is actually a drop in infectivity, presumably due to the invasion of fresh susceptible cells and disappearance of virus in them. This lag is followed by another rise in titre.

(*e*) The highest titre is independent of the initial dose (within the range being discussed) and is about 10^{11} LD 50/g. liver.

(*f*) The efficiency of virus multiplication, expressed as the titre at any time divided by the 5 min. titre, is fairly constant over the range tested, i.e. there is little auto-interference.

DISCUSSION

Two differences can be seen between this host-virus system and others. (*a*) Fourteen infectious units (LD 50's) can multiply in one cell and produce the same final titre as does one LD 50. (*b*) The increases in any one cell follow a stepwise pattern. Both these findings strongly suggest that in this system, the fundamental virus-synthesizing centres must be sought at a subcellular level.

In this connexion, the findings of Watson (1952) are of great interest. She studied the multiplication of mumps virus in the embryonated egg, using specific staining with fluorescein-labelled immune serum and found that the staining seen was such as to suggest multiple foci of infection within each cell. Ackermann & Kurtz (1952) and Schwerdt & Pardee (1952) suggested that such virus-synthesizing foci may be mitochondria. Preliminary experiments with ectromelia virus have shown that infectivity of the order of one-tenth of the total, and no detectable complement-fixing antigen, is present on mitochondria, but this does not necessarily mean that they are not the site of virus production.

The finding that the titre after the first increase corresponds to the original infecting titre suggests two possibilities which will be considered in more detail. First, the eclipse period could represent a breaking up of virus into smaller parts which are non-infective. A proportion only of infectious units, say 1 in 30, yields units effective in initiating multiplication, which starts in the eclipse period, so that at the end of the first rise, with a thirty-fold multiplication of effective units, the 5 min. titre is regained. If the above process took place with an original titre of 10^8, that is about one LD50/cell, and if only one in thirty of the infecting units multiplied, only one cell in thirty would show evidence of infection. That this is not found is shown in Table 1 where the number of glycogen-deficient cells is found to be about one-third to one-half of the injected dose, and Table 2 shows a similar ratio between titre at 5 min. and injected dose, indicating 1 to 1·5 glycogen deficient cells/infectious unit in the liver at 5 min. Unless there is multiplication in some cells, and not in others (although all show the same histological changes), it is difficult to regard the first decrease and increase in titre as simple multiplication.

Another possibility is that the first decrease and increase in titre does not represent multiplication but is a preliminary step to establishing control over some of the synthetic functions of the host. This would involve either a non-infectious host-virus complex without actual breakdown of the virus, infectivity returning as the host-virus complex dissociated, or there could be a temporary break-up of the virus particle. This preliminary step should condition one or more of the cells' synthetic centres, so that after a lag period, multiplication of a stepwise nature could take place. Each centre appears to be exhausted by the production of some thirty particles, which then initiate similar processes on further centres.

This host-virus system differs from others studied at comparable dosage levels, but the differences may not be so great as appears at first sight. Continuing virus production after the initial increase, with or without obvious steps, may be a function of the type of host cell, rather than of the virus and may be more obvious in liver than in other cells. The preliminary period without overall multiplication could exist in other systems but, if it occupied a smaller proportion of the time between infection and death of the cell, it would not be obvious.

This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

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(*Received* 20 *July* 1953)

HOCKENHULL, D. J. D., FANTES, K. H., HERBERT, M. & WHITEHEAD, B. (1954). *J. gen. Microbiol.* **10**, 353–370.

Glucose utilization by *Streptomyces griseus*

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SUMMARY: Glucose utilization by a strain of *Streptomyces griseus* was studied. Under highly aerobic conditions, glucose was converted mainly to structural material and CO_2, but under restricted aeration, lactic acid was formed. Pyruvic acid was also formed during the stages of most rapid growth. The metabolism of glucose was dependent upon the presence of phosphate, and the optimal hydrogen-ion concentration for both glucose oxidation and the rate of disappearance of inorganic phosphate was about pH 7. Phosphate esters, tentatively identified as glucose-1-phosphate and glucose-6-phosphate, were obtained in fluoride-inhibited systems. Glucose oxidation was depressed by 10^{-3}M-sodium iodoacetate and 10^{-2}M-sodium arsenite but was stimulated by 10^{-2}M-sodium arsenate; 10^{-3}M-2 : 4-dinitrophenol and 10^{-3}M-sodium azide had no effect. Streptomycin production was decreased by 3×10^{-3}M-sodium arsenate but not by 10^{-2}M-sodium fluoride or 10^{-2}M-sodium iodoacetate. *S. griseus* metabolized members of the tricarboxylic acid cycle, although citrate and α-ketoglutarate gave much lower values of Q_{O_2} at pH 7·3 than pyruvate, acetate, succinate, fumarate or malate. Ketoacids were produced in presence of arsenite from fumarate, malate, glucose, lactate, acetate, succinate, glutamate and citrate in descending order of yield. Except from fumarate, which yielded some material behaving like α-ketoglutarate, the product was chiefly pyruvate.

Much of the literature on actinomycete metabolism, including the relationship between carbohydrate utilization, hydrogen-ion concentration and mould growth, was reviewed by Waksman (1950). Surveys of the availability of carbon from various carbohydrates were made for a wide range of organisms (Pridham & Gottlieb, 1948), for *Streptomyces coelicolor* (Conn, 1943) and for *S. griseus* in connexion with streptomycin production (Dulaney & Perlman, 1947; Dulaney, Hodges & Perlman, 1947; Dulaney, 1949). The last-mentioned author divided the fermentation into two phases, crescence and senescence. In the former there was uptake of soluble nitrogen, carbon and phosphate into the mycelium, high oxygen demand and rapid utilization of glucose, but very little antibiotic production; in the latter phase, mycelial weight declined, phosphate and nitrogen were excreted into the medium, oxygen demand fell and streptomycin was produced. There was some formation of lactate early in fermentation, but this disappeared rapidly. Similar results were obtained by Woodruff & Ruger (1948). Typical fermentation diagrams were included in a later paper by Perlman & Wagman (1952). The pH curve obtained by these authors for fermentations on glucose-containing media is typical not only of most aerobic *S. griseus* fermentations, but also for most *Streptomyces* spp. under similar conditions. The last-mentioned paper described the effect of replacing glucose by various lipid materials, and showed that growth and streptomycin production were maintained in a medium based upon soybean meal when all the glucose was replaced by soybean, cotton-seed or coconut oil. It was also shown that addition of extra phosphate to the glucose medium depressed streptomycin production and at the same time increased the rate of sugar disappearance. Addition of phosphate to an oil-containing medium depressed antibiotic production to a similar extent but caused no change in rate of lipid utilization. Without giving any figures, the authors state that phosphate increased the Q_{O_2} of the mould in presence of glucose or glycerol, but not in presence of lipids.

An attempt is made here to present a picture of the metabolism of glucose by a strain of *S. griseus* and to relate the findings to pathways of phosphorolytic breakdown such as the Embden-Meyerhof scheme.

METHODS

Organism and cultural conditions. The organism, *S. griseus* albus mutant (Dulaney Z38), is described in the patent covering its production (Dulaney, 1951). The inoculum was grown for 48 hr. at 28° with shaking on the following medium: glucose 10, dried enzymic digest of casein 10, sodium chloride 10, meat extract 6, g./l. distilled water. This was then used to inoculate 40 ml. of the following medium in 250 ml. conical flasks: glucose 25, soybean meal 40, sodium chloride 2·5, distillers' dried solubles 5, g./l. distilled water (pH before autoclaving at 15 lb./sq.in. steam for 20 min., 7·3–7·5). A defined medium, 'Lumb's medium' (Chesters & Rolinson, 1951) was also used. This was based on glucose, glycine, sodium citrate and inorganic salts.

Preparation of mycelial suspensions. Mycelium from 48 hr. cultures (1 vol. medium) was washed 3 times with 1 vol. 0·01 M-potassium phosphate pH 7·0, and re-suspended in 1 vol. of similar buffer. After incubating with shaking for 24 hr. at 28·5°, it was again washed thrice with buffer before resuspension in similar buffer for experimentation. The method of washing and starving is similar to that described for *Penicillium chrysogenum* (Hockenhull, Walker, Wilkin & Winder, 1952).

Although growth was sometimes abnormally rapid, maximal mycelial weight was usually achieved on Lumb's medium after 2–3 days. Maximal respiratory activity was manifest at 1 day.

The Q_{O_2} value for whole broth was highest at 24 hr., fell slowly to 48 hr. after which it declined rapidly to 50% in 72 hr., while that for washed mycelium with respect to glucose had fallen only about 15% in the same time (Table 1; see Gottlieb & Anderson, 1948).

Manometric measurements. Oxygen uptake was measured using Warburg manometers with single sidebulb cups fitted with centre-wells. The temperature was 28·5° and the gas phase was air. The results obtained are expressed as $Q_{O_2} = \mu l.\ O_2$ consumed/mg. dry-wt. mycelium/hr. When the effect of various treatments, e.g. the use of inhibitors, was investigated the assumption was made that there was no interaction between the action of substrate and inhibitor upon the endogenous respiration. The respiration due to substrate in presence of inhibitor was corrected by subtraction of the endogenous respiration in presence of inhibitor. Attention is drawn to the arbitrary nature of this proceeding.

Table 1. *Respiratory activity of* S. griseus *at different ages*

S. griseus grown in defined medium (40 ml. medium/250 ml. flask) with shaking at 28·5°. Sets of three replicate flasks harvested daily. Washing and starving carried out by the standard procedure with 3 × 1 vol. 0·01 M-phosphate pH 7·0. Warburg manometers contained: *in main compartment*, 2 ml. mycelial suspension; *in side bulb*, 0·2 ml. 0·01 M-phosphate buffer, with or without 0·1 M-glucose; *in centre well*, 0·1 ml. 10 N-KOH on filter-paper. Measurements taken at 28·5° over 30 min.

					Washed mycelium, Q_{O_2}			Washed starved mycelium, Q_{O_2}		
Day	Mycelial wt. (mg./ml.)	pH	Streptomycin (mg./l.)	Whole broth Q_{O_2}	A (total)	B (endogenous)	A − B (glucose)	C (total)	D (endogenous)	C − D (glucose)
0	0·017	6·24	0	—	—	—	—	—	—	—
1	0·75	6·94	0	—	45·6	18·6	29·2	—	—	—
				48·0	48·8	19·2				
				48·8	50·8	19·7				
2	3·43	7·78	0	43·7	43·6	17·5	26·4	28·4	10·1	18·3
				43·1	42·9	18·0		28·8	9·9	
				42·7	42·8	17·6		27·8		
3	11·6	8·10	215	23·9	40·0	16·2	24·75	16·4	6·75	9·8
				24·0	41·6	15·9		16·5	6·65	
4	10·05	8·50	245	10·1	14·8	8·3	6·6	9·6	3·5	6·1
				10·1	15·0	8·3		9·8	3·7	
5	10·25	8·96	220	5·05	9·85	4·15	5·5	8·2	2·3	5·95
				5·0	9·45	4·15		8·4	2·6	
6	10·35	8·98	240	2·8	8·3	3·2	5·15	—	—	—
				2·6	8·4	3·1				

Analytical procedures

Carbohydrates. Total reducing sugar was determined by the method of Somogyi (1937). Reducing sugars after hydrolysis (expressed as glucose) were determined by the same method after heating in 0·8 N-HCl at 120° for 30 min. and neutralizing with NaOH. Qualitative examination of carbohydrate materials was carried out chromatographically by descending flow on Whatman No. 4 paper. The solvents most frequently used were *n*-butanol/pyridine/water mixtures, 3/2/1·5 or 4/1/1 by volume (Jeanes, Wise & Dimler, 1951). Carbohydrates were located by spraying with acid naphthoresorcinol (Bryson & Mitchell, 1951) or methanolic borate-phenol red (Hockenhull, 1953). Aminosugars were located by a spray (Partridge, 1949) based on the reaction of Elson & Morgan (1933).

Lactate was determined by the method of Gordon & Quastel (1939) which is claimed not to suffer interference from pyruvate. *Carboxylic acids* were separated by paper chromatography (Lugg & Overell, 1947; Stark, Goodban & Owens, 1951), as also were *amino acids* using the solvent systems of Partridge (1948). *Keto-acids* were estimated by the method of Friedemann & Haugen

(1943). They were separated by paper chromatography of the 2 : 4-dinitrophenylhydrazones (Cavallini, Frontali & Toschi, 1949) or of the nitroquinoxalinols formed by condensation of α-ketoacids with 1 : 2-diamino-4-nitrobenzene (Hockenhull & Floodgate, 1952; Hockenhull, Hunter & Herbert, 1953). Larger quantities of these derivatives were separated by chromatography on alumina columns (Datta, Harris & Rees, 1950; Hockenhull, Herbert, Walker, Wilkin & Winder, 1954; Hockenhull & Floodgate, 1952).

Streptomycin was determined as maltol (Boxer, Jelinek & Leghorn, 1947).

Phosphate was determined by the method of Fiske & Subbarow (1925). *Sugar phosphates* were fractionated by the method of Vercellone & Neuberg (1935). *Fructose* was estimated by the method of Roe (1934). *Phosphate esters* were separated chromatographically by ascending flow on Whatman no. 4 paper using the solvent systems methanol/formic acid/water (80/15/5, v/v) and methanol/ammonia (sp.gr. 0·88)/water (60/10/30, v/v) (Bandurski & Axelrod, 1951).

RESULTS

Changes in the carbohydrate content of Streptomyces griseus *cultures*

Changes in soybean medium. Soybean medium contains glucose as the principal carbohydrate with certain carbohydrates from the soybean meal—sucrose, raffinose, stachyose (Street & Bailey, 1915; Sato, 1921). After sterilization some material resembling fructose is present. Cultures were harvested in triplicate at regular intervals and the residual carbohydrate determined (Table 2).

The initial non-reducing sugar, averaging 3·6 mg./ml. as glucose, was accountable for by the non-utilizable sugars of soybean meal, sucrose, raffinose and stachyose (Pridham & Gottlieb, 1948). A rise after 2 days to about 6 mg./ml. indicated a synthesis of some other hydrolysable carbohydrate material which was of a transient nature, the total falling to about 4·0 mg./ml. at 3 days. In the later stages of growth, the hydrolysable carbohydrate rose to about 8 mg./ml., implying a considerable synthesis of sugar polymers. Paper chromatographic separation of the sugars from a similar fermentation revealed no identifiable carbohydrates other than sucrose, raffinose, stachyose, glucose and a material similar to fructose which was possibly derived from glucose by isomerization during sterilization. The three non-utilizable sugars were also separated by means of charcoal columns (Whistler & Durso, 1950), but no new substances synthesized during growth could be detected.

Changes in Lumb's medium. Table 3 shows the amount of reducing sugar in filtrates from cultures of *S. griseus* growing on Lumb's medium. Very little more reducing sugar appeared after acid hydrolysis, indicating little polymerization of sugars. Although the medium contained glucose as sole carbohydrate before autoclaving, a number of new substances reacting like carbohydrate appeared after heat treatment. A material which behaved chromatographically like, and presumably was, fructose (possibly formed by a Lobry de Bruyn isomerization), disappeared at roughly the same rate as glucose. A number of spots which became red or purple on treatment with naphthoresorcinol were also formed. The substances corresponding to these were not utilized, and probably represented only a small proportion of the

Table 2. *Sugar content of soybean medium during growth of* S. griseus

Cultures on soybean medium grown as described by Dulaney (1951) at 28·5° with shaking. Flasks removed daily in triplicate for determinations.

Day	Reducing sugar (mg./ml.)	Total sugar after acid hydrolysis (mg./ml.)	Non-reducing sugar (mg./ml.)
0	26·0	29·9	3·90
	25·7	29·7	3·95
	25·9	28·9	3·00
1	25·3	29·7	4·40
	24·8	29·5	4·70
	25·3	29·0	3·75
2	14·4	21·7	7·25
	17·0	22·2	5·15
	14·7	20·5	5·80
3	10·2	14·0	3·85
	10·9	14·5	4·60
	11·3	14·8	3·45
4	7·00	12·65	5·65
	7·10	12·65	5·55
	7·87	12·72	5·85
5	2·70	9·95	7·25
	2·40	10·00	7·60
	2·25	10·10	7·85
6	1·50	10·00	8·50
	1·50	8·50	7·00
	0·67	8·50	7·83
7	1·25	9·50	8·25
	1·25	8·75	7·50
	1·25	9·50	8·25
8	1·25	9·50	8·25
	1·25	8·40	7·15
	1·25	8·35	7·10

Table 3. *Changes in carbohydrate content of Lumb's medium during growth of* S. griseus

Cultures grown at 28·5° with shaking. Flasks removed in triplicate and pooled for analysis.

	Day								
	0	1	2	3	4	5	6	7	8
	Reducing sugar (mg./ml.)								
Before hydrolysis	17·7	19·3	13·7	3·0	0·42	0·40	0·34	0·37	0·29
After acid hydrolysis	16·3	17·7	13·6	2·7	0·62	0·50	0·57	0·49	0·37

total carbohydrate. Autoclaving the medium constituents in pairs at similar concentrations to those in Lumb's medium led to formation of substances which reacted with acid naphthoresorcinol from (*a*) glycine heated alone, and (*b*) by interaction between glucose or citrate and the trace minerals (Table 4). The spots from glycine may have been due to its reaction with cellulose on drying or treating the paper with acid naphthoresorcinol.

Table 4. *Chromatographic behaviour of autoclaved mixtures of components of Lumb's medium at concentration and* pH *value of medium*

R_G is defined as the ratio of the distance moved by the spot to the distance moved by glucose.

Medium component	Colour or nature of spot	Distance moved (cm.) in 16 hr. (butanol/pyridine/water (3 : 2 : 1·5)	Approximate R_G
Glucose (A)	Glucose and fructose spots	Glucose 22, fructose 27	—
Glycine (B)	Reddish pink	1	0·05
Na-citrate (C)	Nil	—	—
Inorganic salts (D)	Nil	—	—
A+B	Glucose, fructose and glycine spots	—	—
A+C	Glucose and fructose spots	—	—
A+D	Two purple spots, one pinkish spot, glucose and fructose	Purple spots 3, 7·5; pink spot 4·5	0·14, 0·34, 0·20
B+D	As B	—	—
C+D	Pink spot	Nil	—

Further experiments, not yet satisfactorily concluded, were carried out to determine the relative amounts of various carbohydrate fractions in the mycelium. The method used, that of Trevelyan, Forrest & Harrison (1952), was subject to considerable sampling error, different sugars varied in extent of reaction, and the fractionation procedures appeared to affect the amount of colour given with the reagent. It was noteworthy, however, that there was present no carbohydrate insoluble in 30% KOH. A typical set of results on 120 ml. of a 4-day culture on Lumb's medium gave: total mycelial dry weight 465 mg.; total anthrone value 47 mg. glucose equivalent, fraction soluble in 10% trichloroacetic acid 28 mg. glucose equivalent, residue after 30% KOH extraction, nil; 59 mg. total mycelial nitrogen (which would correspond, ignoring amino-sugars and other non-protein nitrogen, to about 360 mg. protein).

The mechanism of glucose utilization

Effect of phosphate level upon Q_{O_2} (glucose). Preliminary work with mycelia washed and starved in water instead of phosphate buffer, indicated that glucose, fructose, mannose, glucose-1-phosphate and hexose diphosphate gave closely similar Q_{O_2} values, whereas starch and gluconate were very much less easily oxidized (Table 5). The effect of phosphate was unusually small, presumably because the cells were still replete with inorganic phosphate. The above experiment was exceptional; in all other experiments in which starving and washing were carried out in water, it was found that inorganic phosphate (pH 7·3) profoundly stimulated the respiration in presence of glucose (Table 6). Orthophosphate could not be replaced by either glucose-1-phosphate or hexose diphosphate.

Table 5. *Oxygen uptake of* S. griseus *in presence of substrates related to the Embden Meyerhof scheme*

Warburg manometers contained: *main compartment*, 9·8 mg. dry-wt. mycelium in 2 ml. water; *side bulb*, 0·2 ml. 0·01 M substrate; *centre well*, 0·1 ml. 10 N-KOH with filter-paper. Temp. 28·5°. Gas phase, air. The pH of substrates and suspensions were adjusted to pH 7·2.

Substrate	Q_{O_2} (corrected for endogenous)	Q_{O_2} in presence of 0·01 M-phosphate pH 7·2 (corrected for endogenous)
Endogenous	7·2	20·2
Glucose	17·1	18·6
Fructose	15·8	16·4
Mannose	16·1	18·2
Glucose-1-phosphate	17·0	—
Hexose diphosphate	16·8	21·7
Starch	1·6	3·9
Sodium gluconate	6·8	—

Table 6. *The effect of orthophosphate and sugar phosphates upon oxygen uptake by* S. griseus *in presence of glucose*

Warburg manometers contained: *main compartment*, about 12 mg. dry-wt. starved mycelium in 2 ml. water, pH 7·0; *side bulb*, 0·1 M substrate in 0·2 ml.; *centre well*, 10 N-KOH with filter-paper. Temp. 28·5°.

Glucose	Ortho-phosphate	Hexose diphosphate	Glucose-1-phosphate		Q_{O_2} (mean increase over endogenous)
Final concentration in mixture				Q_{O_2}	
0	0	0	0	11·5 11·5	—
0·01 M	0	0	0	28·7 28·6	17·2
0	0·01 M	0	0	17·0 17·2	5·6
0·01 M	0·01 M	0	0	52·1 52·3	40·7
0·01 M	0	0·01 M	0	26·7 28·2	15·8
0·01 M	0	0	0·01 M	27·9 28·2	16·6

Effect of phosphate upon glucose disappearance. Suspensions of *S. griseus* washed and starved in water were incubated at 28·5° with shaking, in presence of glucose, and various concentrations of phosphate buffer pH 7·3. Glucose utilization increased with rising phosphate concentration (Table 7), a result fully consistent with the respirometric findings. It should be noted, however, that the pH optima for both glucose and phosphate utilization are extremely sharp. Although the initial pH was the same in all treatments (pH 7·3) the final pH may have risen to values giving faster rates of utilization where more phosphate was present. The effects were, however, somewhat larger than would have been expected if pH change was the whole reason.

Isolation of phosphorylated intermediates. Attempts to trap triosephosphates as the phenylhydrazine derivative of methylglyoxal, on similar lines to the work of Scevola & Valcurone (1952), proved unsuccessful, although traces of pyruvate were noted. Consequently an attempt was made to obtain phosphate esters by the incubation of mycelium with glucose and phosphate in presence of fluoride. The technique used was similar to that of Neuberg & Kobel (1933).

Table 7. *Effect of phosphate concentration on glucose utilization*

232 mg. dry-wt. mycelium in 20 ml. of glucose solution 1·64 mg./ml., with varying levels of phosphate, shaken in 250 ml. conical flasks for 4 hr. at 28·5°.

Added K-phosphate pH 7·3 (M)	Glucose utilized (mg./ml.)
0	0·62
0·001	0·92
0·002	1·24
0·01	1·64
0·02	1·58
0·10	1·50

A qualitative experiment was carried out with mycelium from 6 l. of 24 hr. cultures of *S. griseus* in meat medium (about 50 g. dry matter). After washing, this was re-suspended in a mixture of 100 ml. 0·1 M-potassium phosphate pH 7·0, 100 ml. 20 % (w/v) glucose, 100 ml. 0·3 M-sodium fluoride, 20 ml. 10 % (v/v) acetaldehyde and water to a total volume of 1 l., the pH value being readjusted to pH 7·0. The whole was apportioned in 60 ml. lots in 200 ml. conical flasks and agitated aerobically at 28·5° for 18 hr. After incubation it was worked up by the technique of Neuberg & Kobel (1933). The yield of water-soluble barium phosphate esters was low (69 mg. phosphorus), and consisted of acid hydrolysable phosphate (glucose 1-phosphate fraction) 42 %, alkali hydrolysable 30 %, hexose-diphosphate 0 % and glucose 6-phosphate (by difference) 28 %. The presence of glucose-1-phosphate and glucose-6-phosphate was confirmed by paper chromatography (Table 8), but no other

Table 8. *Chromatographic separation of phosphate esters*

Water-soluble barium salts, obtained by fluoride-inhibited metabolism of glucose, examined by the technique of Bandurski & Axelrod (1951). The solvent fronts in both systems moved about 20 cm. in 2 hr.

	R_F	
	Methanol/formic acid/water (80 : 15 : 5)	Methanol/0·880 sp.gr. ammonia/water (60 : 10 : 30)
Standard substances		
Glucose-1-phosphate	0·16	0·62
Glucose-6-phosphate	0·39	0·36
Hexose diphosphate	0·42	0·25
Orthophosphate	0·65	0·28
Ba esters soluble in water	0·16	0·64
	0·36	0·37
	0·63	0·27

esters were identified. No hexose-diphosphate could be detected in the water insoluble phosphate fraction. No esters were detectable in samples taken at the commencement of incubation.

A smaller experiment was carried out on the same lines as above, using mycelial suspensions from cultures on Lumb's medium (1·6 g. dry wt.) in volumes of 50 ml. Fluoride was omitted from one flask. The water soluble barium salts, which gave a similar pattern on chromatography, amounted to 0·281 g. with sodium fluoride and 0·132 g. without sodium fluoride.

Dependence of glucose oxidation on hydrogen-ion concentration. Washed starved mycelial suspensions were incubated with 0·01 M-glucose in presence of 0·1 M-potassium phosphate buffer at various pH values. The suspension was made in similar buffer, which was adjusted to the correct pH with N-HCl or N-KOH. Fig. 1 indicates the dependence of respiration on pH and shows that the maximum lies near pH 7·0.

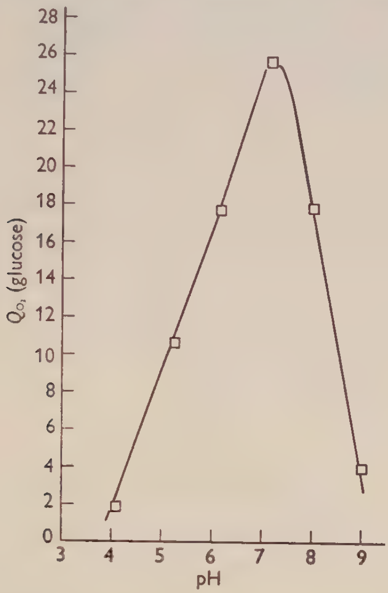

Fig. 1. Effect of hydrogen-ion concentration upon oxygen uptake in presence of glucose. Warburg manometers contained: *main compartment*, 5·6 mg. dry-wt. starved mycelium in 2 ml. 0·1 M-K phosphate buffer at appropriate pH; *side bulb*, 0·2 ml. 0·1 M-glucose in 0·1 M-K phosphate at appropriate pH; *centre well*, 0·1 ml. 10 N-KOH with filter-paper. Temp. 28·5°. Gas phase, air.

Effect of hydrogen-ion concentration upon phosphate utilization. Mycelium from 48 hr. cultures of *S. griseus* was spun down and washed 4 times with distilled water and once with 0·05 M-barbiturate buffer, pH 7·02. KH_2PO_4 (0·080 g.) was dissolved in 20 ml. 0·05 M-barbiturate buffer and added to the mycelium resuspended in 200 ml. of buffer containing 0·01 M-glucose. The pH value of 20 ml. portions in 250 ml. conical flasks was adjusted with conc. HCl or 10 N-NaOH, to avoid dilution. Flasks were shaken aerobically at 28·5° for 4 hr. after taking 2 ml. from each for phosphate determination, which was repeated at the end of the 4 hr. Fig. 2 shows the relationship between mean pH over the 4 hr. of the experiment and ortho-phosphate disappearing. Maximal phosphate disappearance took place between pH 7·0 and 7·5, this maximum corresponding with that for glucose utilization as measured by the Q_{O_2} (glucose) value. Care is necessary in interpreting this experiment as there was a considerable shift in pH. This has been compensated for in Fig. 3 by using the mean value. In the experiment giving a mean of 9·00, the pH moved from 9·52 to 8·48, in that giving a mean of 6·28 from pH 5·48 to 7·08, in the experiment showing an initial value of 5·12, pH did not change much (5·02–5·25), and at this value phosphorylation, very significantly, fell almost to zero. The initial value at which there was no change was 8·02. Another similar set of results is embodied in Fig. 2.

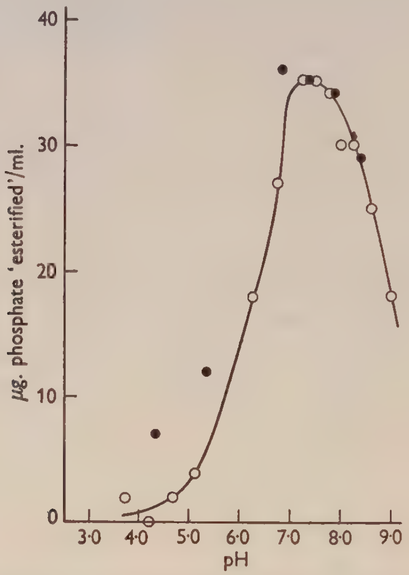

Fig. 2. Effect of hydrogen-ion concentration upon the esterification of inorganic phosphate. 250 ml. conical flasks containing 82 μg. orthophosphate P/ml. in 20 ml. 0·05 M-barbiturate buffer at various pH values with 0·01 M-glucose and 13·1 mg. dry weight mycelium/ml. Shaken for 4 hr. at 28·5°. The two sets of points represent parallel experiments with similarly prepared mycelium.

Anaerobic metabolism

Production of lactic acid in Lumb's medium under conditions of restricted aeration. Cultures of *S. griseus* were grown on Lumb's medium with shaking. After 24 hr. aerobic growth, during which time a considerable mycelial weight had been produced, half of the flasks were fitted with tight rubber caps to exclude air. At regular intervals, flasks were removed in triplicate, lactic acid, glucose and dry matter were determined, and their mean values were plotted against time of incubation (Fig. 3*a*, *b*). There was very little change in metabolism during the early stages, when presumably sufficient oxygen for respiration remained in the capped flask. However, the mycelial weight increased much more slowly under restricted aeration, so that the rate of glucose utilization/mg. of dry matter was higher under anaerobic conditions. Although there was a moderate quantity of lactic acid in both series by the second day, this increased to the high value of 5·6 mg./ml. by the third day in the poorly aerated series. This is what might be expected in a system such as that of Embden and Meyerhof in anaerobiosis.

Anaerobic dehydrogenation of hexose diphosphate and glucose. An experiment carried out at pH 7·2 in 0·125 M-$NaHCO_3$ in equilibrium with a 5 % CO_2–95 % N_2 gas mixture gave the following Q_{CO_2} values (corrected for endogenous respiration); endogenous 4·70, 0·01 M-glucose 0·03, 0·01 M-hexose diphosphate 1·9 and, in presence of 0·1 % $K_3Fe(CN)_6$ as hydrogen acceptor, glucose 35·6, hexose diphosphate 9·4. The Q_{O_2} (endogenous) was 5·6 and Q_{O_2} (glucose, corrected for this endogenous value) was 41·6 on the same suspension.

Inhibitor studies

The effect of various inhibitors upon Q_{O_2} *(glucose).* The respiration of washed suspensions of *S. griseus* in phosphate buffer pH 7·0 was determined in presence of various inhibitors. Table 9 shows clearly that considerable inhibition was caused by 0·001 M-sodium iodoacetate and by 0·01 M-sodium arsenite. Iodoacetate, however, appeared to increase endogenous respiration. 2 : 4-Dinitrophenol increased endogenous respiration. Azide (0·001 M) had little or no stimulatory effect. Arsenate, however, gave considerable stimula-

Table 9. *Effect of inhibitors on* Q_{O_2} *(glucose)*

Warburg manometers contained: *main compartment*, 6·9 mg. dry-wt. mycelium in 2 ml. 0·01 M-K phosphate (pH 7·0) containing inhibitor at concentration indicated; *side bulb*, 0·2 ml. 0·1 M-glucose; *centre well*, 0·1 ml. 10 N-KOH with filter-paper. Temp. 28·5°.

		Q_{O_2}		% stimulation (+) or inhibition (−) of respiration	
Expt.	Inhibitor M	Endogenous	Corrected glucose	Endogenous	Corrected glucose
1	0	8·7	18·3	0	0
	2 : 4-dinitrophenol, 0·001	16·1	26·3	+85	+ 6
	NaN_3, 0·001	10·4	22·8	+20	+29
	Na iodoacetate, 0·001	13·8	16·4	+59	−73
	Na arsenite, 0·01	5·8	9·9	−33	−57
2	0	10·9	23·8	0	0
	2 : 4-dinitrophenol, 0·001	19·1	31·2	+75	− 6
	NaN_3, 0·001	10·3	23·9	− 6	+ 6
	NaF, 0·004	9·3	21·7	−15	− 3
	Na arsenate, 0·01	9·7	26·8	−11	+33
3	0	10·1	20·6	0	0
	Na iodoacetate, 0·001	12·0	18·4	+19	−39
	Na arsenite, 0·01	3·0	6·5	−70	−67

tion of respiration, as might be expected by analogy with recorded instances of glycolysis in yeast. Preliminary experiments indicated that anaerobic CO_2 production from glucose or hexose diphosphate was stimulated by arsenate.

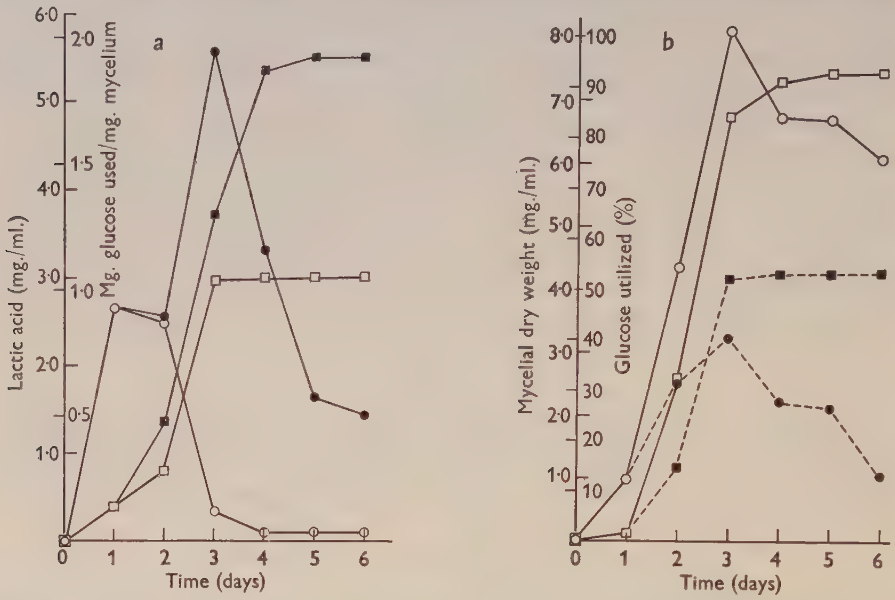

Fig. 3. Utilization of glucose and production of lactic acid in Lumb's medium. Mycelium grown aerobically for 24 hr. in shaken culture at 28·5° using 40 ml. Lumb's synthetic medium/250 ml. flask. At 24 hr. half of flasks capped to exclude oxygen. At intervals flasks removed in triplicate for analysis. *a*, ●—●, lactate anaerobic; ○—○, lactate aerobic; ■—■ mg. of glucose utilized/mg. dry wt. of mycelium, anaerobic; □—□, mg. of glucose utilized/mg. dry wt. of mycelium, aerobic. *b*, ●---●, mycelial dry wt., anaerobic, ○—○, mycelial dry wt., aerobic; ■—■, percentage total glucose utilized, anaerobic; □—□, percentage total glucose utilized, aerobic.

The effect of various inhibitors upon growing cultures. Iodoacetate at concentrations from 10^{-4}M to 5×10^{-2}M had no discernible effect on growth or streptomycin production on soybean media. A similar experiment showed that 2×10^{-5}M to 10^{-2}M-sodium fluoride had no detectable effect on growth or streptomycin titre.

Sodium arsenate, which had already been shown to increase the rate of oxygen uptake in presence of glucose, was also found (Table 10) to decrease the streptomycin titre without any apparent effect upon growth. (The latter could only be estimated very roughly owing to the high solids content of the medium.) However, arsenate added early in the fermentation had a secondary effect in bringing about acid pH conditions which may also have contributed towards decreasing the yield of streptomycin.

Metabolism of acids related to the tricarboxylic acid cycle

Oxygen uptake in presence of organic acids. Suspensions of washed, starved mycelium were tested with a number of substrates at various pH values (Table 11). At pH 7·0, the highest respiration rate was obtained with glucose.

Table 10. *Effect of phosphate or arsenate on streptomycin yield*

Fermentations of soybean medium (40 ml./250 ml. shaken flask) carried out according to Dulaney (1951, Expt. 1) at 28·5°. Seitz-filtered arsenate or phosphate adjusted to pH 7·0 was added to give the strength quoted in the table at the time indicated. Flasks were withdrawn in triplicate for pH determination and streptomycin assay (Boxer, Jelinek & Leghorn, 1947).

Expt.	Addition	Time of addition (day)	Streptomycin titre (μg./ml.)		pH value	
			Day 6	Day 7	Day 6	Day 7
1	Water (control)	—	—	1350	—	7·10
			—	1180	—	7·62
			—	1310	—	7·44
1	K phosphate, 0·015 M	0	—	580	—	8·08
			—	510	—	8·08
			—	420	—	8·06
2	Water (control)	—	1110	1120	7·98	8·28
			1280	1020	7·94	8·26
			1360	980	7·82	8·28
2	Na arsenate, 0·003 M	4	680	645	7·76	7·22
			770	640	7·70	7·62
			750	595	7·62	7·48
2	Na arsenate, 0·003 M	3	350	310	7·58	8·24
			250	290	7·68	8·38
			330	330	7·78	8·10
2	Na arsenate, 0·003 M	2	15	10	5·82	5·88
			15	15	5·80	5·86
			10	10	5·74	5·88

Table 11. *Q_{O_2} values at various hydrogen-ion concentrations for substances related to the tricarboxylic acid cycle*

Warburg manometers contained: *main compartment*, 2·0 ml. washed starved 48 hr. mycelium in 0·1 M-K phosphate at appropriate pH; *side bulb*, 0·2 ml. 0·1 M substrate adjusted to same pH; *centre well*, 10 N-KOH on filter-paper. Temp. 28·5°. The Q_{O_2} (substrate) values have been corrected for Q_{O_2} (endogenous).

Substrate	Expt.	mg. dry-wt. mycelium/Warburg flask	Q_{O_2}					
			pH 5·0 ± 0·1		pH 7·0 ± 0·1		pH 8·0 ± 0·1	
			Endogenous	Substrate	Endogenous	Substrate	Endogenous	Substrate
Glucose	1	5·6	6·1	3·55	13·6	17·7	16·1	17·3
Lactate	2	6·5	5·9	11·6	6·6	7·9	9·4	2·3
Pyruvate	3	4·9	4·4	10·9	8·3	12·2	11·7	8·6
Acetate	3	4·9	4·4	4·4	8·3	10·6	11·7	9·0
Citrate	4	4·7	7·2	0·35	15·7	2·2	14·1	1·5
Glutamate	4	4·7	7·2	7·0	15·7	8·0	14·1	5·2
α-Ketoglutarate	5	6·0	3·6	5·1	9·4	4·6	10·4	2·8
Succinate	6	8·2	4·2	5·3	10·4	9·2	10·6	4·9
Fumarate	7	7·1	8·6	4·8	10·1	12·3	12·3	10·5
Malate	8	8·2	5·1	6·2	9·3	7·3	11·7	5·8

Fumarate, pyruvate, acetate, succinate, lactate, glutamate and malate were utilized fairly readily, but α-ketoglutarate and citrate gave only a small increase in oxygen consumption. Lowering the pH to 5·0 decreased most Q_{O_2} values (glucose, acetate, citrate, succinate and fumarate), those of pyruvate, α-ketoglutarate and malate remained substantially the same, and that of lactate rose by about 40 %. Raising the pH to 8·0 led to a severe fall in Q_{O_2} values for lactate and a moderate fall for pyruvate, citrate, α-ketoglutarate, glutamate, succinate and malate, those of glucose, acetate and fumarate remaining substantially the same.

Production of keto-acids in S. griseus *cultures.* Our colleagues, Dr M. McGonagle and Miss P. Dayton, drew our attention to the presence of keto-acids in certain growing cultures, and it was confirmed that young (20–30 hr.) cultures contained considerable amounts of keto-acids.

Table 12. *Keto-acid derivatives from* S. griseus *cultures*

All chromatograms carried out on Whatman no. 4 paper by ascending flow. About 10 μg. total derivative used. Distance moved by the solvent front about 25 cm.

	100 × R_F values of 2 : 4-dinitrophenylhydrazones		
Material examined	1 % Na_2CO_3 (2 hr.)	*sec*-butanol/4 % (v/v) aqueous 0·880 sp.gr. ammonia (80 : 20) (17 hr.)	*n*-hexanol satd. with 4 % v/v aqueous 0·880 sp.gr. ammonia (17 hr.)
Authentic			
Dimethyl pyruvic	60	>95	65
α-Ketobutyric	42	>95	48
Pyruvic	59, 42	85, 76	14, 33
Glyoxalic	57	49	—
Oxaloacetic	80	15	0
α-Ketoglutaric	62	9	0
Keto-acids in 24 hr. culture in meat medium	72	>95	89
	57	86	66
		15 (trace)	14
		8 (trace)	
Keto-acids in 24 hr. culture in Lumb's medium	57	74	12
Keto-acids formed on incubating washed, starved mycelium with K fumarate	57	8	14
	62	15 (trace)	66 (trace)
	77 (trace)	77	89 (trace)
Keto-acids formed on incubating washed, starved mycelium with K lactate	57	74	15
Keto-acids formed on incubating washed, starved mycelium with K malate	56	77	13
	65 (trace)	8 (trace)	

Inoculum development (meat) medium cultures, as described by Dulaney (1951), contained 0·38 mg. keto-acid/ml. at 24 hr. This was found to be principally 'pyruvate' as estimated by the method of Friedemann & Haugen (1943), and paper chromatography revealed that about 90 % was pyruvate. There appeared also to be traces of α-ketoglutarate and oxaloacetate, as well as two substances giving faster-moving 2 : 4-dinitrophenylhydrazones (Table 13). The pyruvate derivative was purified by passage through an alumina column and recrystallized, m.p. 217–218·5° (authentic compound m.p. 217–217·5°; mixed m.p. 217–218·5° uncorr.). From another portion of the same culture fluid was prepared 3-methylnitroquinoxalin-(2)-ol, m.p. 246–249° (authentic compound m.p. 247–248°; mixed m.p. 246–249° uncorr.).

Lumb's medium at 24 hr. contained 0·198 mg. keto-acid/ml. (0·162 mg. apparent pyruvate/ml.; Table 12). The 2 : 4-dinitrophenylhydrazone of pyruvate was isolated, m.p. 217–218° (authentic compound, m.p. 217–217·5°; mixed m.p. 217° uncorr.). The keto-acid content of a 24 hr. culture in soybean medium resembled that of meat medium. Again pyruvate was the predominant component and its 2 : 4-dinitrophenylhydrazone was isolated, m.p. 217–218° (authentic compound m.p. 217–218°; mixed m.p. 217·5° uncorr.).

Keto-acid production from organic acids. Washed mycelial suspensions were incubated with various substrates in presence or absence of 0·02 M-sodium arsenite. All experiments were carried out in 0·005 M-potassium phosphate pH 7·2, using the potassium salts of the acids at 4 mg. C/ml. After 4 hr. shaking in air at 28·5°, the flasks were tested for keto-acid production (Table 13). Much higher yields were obtained in the presence of arsenite than in its absence.

Table 13. *Production of keto acids from organic acids and glucose*

250 ml. flasks contained 40 ml. of the following mixture: glucose or K salts of organic acids at a concentration equivalent to 4 mg. carbon/ml. in 0·005 M-K phosphate pH 7·2 containing 0·01 M-Na arsenite and about 140 mg. dry-wt. of washed, starved *S. griseus*. The flasks were shaken for 4 hr. at 25°; gas phase, air. Bracketed figures represent keto-acid titre in absence of arsenite.

	Total keto-acids (mg./ml.)	'Pyruvate' (mg./ml.)	'Ketoglutarate' (mg./ml.)
Glucose	4·50 (0·07)	4·45 (0·02)	0·05 (0·05)
Acetate	2·32 (0·10)	2·32 (0·00)	0·00 (0·10)
Lactate	2·49 (0·00)	2·49 (0·00)	0·00 (0·00)
Glutamate	0·07	0·00	0·07
Citrate	0·03	0·02	0·01
Succinate	1·08	1·12	0·00
Fumarate	22·9 (1·22)	14·3 (0·04)	8·6 (0·82)
Malate	13·9 (0·58)	12·9 (0·23)	1·0 (0·35)
Control	0·04	0·02	0·00

Similar experiments carried out at pH 5·0 and 8·5 without arsenite, or at pH 7·2 with 10^{-3} M-2 : 4-dinitrophenol gave very much lower yields. Pyruvate was the principal keto-acid formed in all cases, the proportion of α-ketoglutaric acid being very small except from fumarate.

The experiment with fumarate was repeated on a larger scale to give a total reaction volume of 320 ml. containing finally about 6·6 mg. keto-acid/ml. (mainly 'pyruvate'). Paper chromatography of the 2 : 4-dinitrophenylhydrazones revealed that pyruvate in both tautomeric forms accounted for most of the keto-acids, but that some α-ketoglutarate and a trace of oxaloacetate were also present (Table 12). The 2 : 4-dinitrophenylhydrazine derivative, isolated from 160 ml. of the reaction mixture on an alumina column, was washed with hot benzene and recrystallized from hot nitrobenzene, m.p. 208–208·5°. Analysis gave C, 40·68; H, 2·98; N, 20·8%—as against C, 40·15; H, 3·30; N, 20·80%—for authentic pyruvic acid 2 : 4-dinitrophenylhydrazone. From a similar 160 ml. of reaction mixture, the nitroquinoxalinols were prepared and isolated. The product was almost all the pyruvate derivative, 3-methyl-nitroquinoxalin-(2)-ol, m.p. 248–248·5° (authentic sample m.p. 247–248°; mixed m.p. 247–248° uncorr.).

It is therefore certain that pyruvate was the principal ketonic product obtained from fumarate or malate. It must be remembered, however, that much of any oxaloacetate originally formed might have been decarboxylated to give this acid under the conditions of isolation. Similar experiments carried out over 24 hr. gave similar yields of keto-acid from lactate or malate; this also was almost wholly pyruvate.

Other constituents of mycelium detected

Mycelium from fermentations of soybean medium or of Lumb's medium was spun down, washed, and hot-water extracts made which were examined for hydroxy and polybasic organic acids. The only carboxylic acid detected in appreciable amount was glutamic acid. Other chromatograms showed that the whole range of amino acids was probably present in addition to glutamic acid, of which about 0·1 mg./ml., was present in the whole culture. The following amino acids were identified at about one-fifth this concentration: alanine, glycine (in the soybean medium; it was present from the start in Lumb's medium), arginine and aspartic acid. About eight other amino acid spots were separable, but were not identified. Apart from glycine in Lumb's medium, which fell to about 0·1 mg./ml., the levels of amino acids remained substantially the same after day 3. It is likely that all amino acids necessarily needed by the organism for its growth can be synthesized.

DISCUSSION

In normal aerobic cultures the principal final products of glucose metabolism are mycelium and CO_2. Under conditions of restricted aeration some accumulation of lactic acid occurs. The results reported do not clearly differentiate between breakdown by way of the classical Embden-Meyerhof scheme or by some alternative mechanism such as the hexose monophosphate shunt (Lipmann, 1936; Dickens, 1938; Koepsell, 1950; Scott & Cohen, 1951). It should, however, be noted that work upon the related organisms *Streptomyces scabies* and *S. coelicolor* (Cochrane, Peck & Harrison, 1953) indicated that the hexose monophosphate shunt is almost certainly the means by which glucose is broken down by these organisms. Although the terminal metabolism cannot be proven to take place by way of a tricarboxylic acid cycle, nothing has been found to support the claims of Garner & Koffler (1951) that it is not operative in *S. griseus*. The failure to metabolize acids of the cycle without prior adaptation, upon which their conclusions are based, may have been due to failure to absorb these acids through the cell wall.

Streptomycin synthesis, the putting together of sugar-like residues, behaved towards arsenate or phosphate (see Perlman & Wagman, 1952) as might be predicted from the behaviour of similar polymerizations (e.g. synthesis of starch from glucose-1-phosphate). This behaviour implies the existence of phosphorylated intermediates. These, however, were not present in detectable amounts.

We wish to thank Mrs D. Goodinson for technical assistance.

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Vitamin requirements of thirty-four Lactic Acid Bacteria associated with Brewery Products

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SUMMARY: The vitamin requirements of thirty-four strains of lactic acid bacteria, representing ten species and isolated from brewery materials, were studied. Individual strains required from one to four vitamins for normal growth on the basal medium used. Pantothenic acid was required by all the organisms. In a number of cases growth failed when groups of vitamins, individually non-essential, were omitted.

As part of a general study of lactic acid bacteria occurring in association with brewery materials, Bhandari, Russell & Walker (1954) isolated sixty-nine strains from sour beers and brewer's yeasts. It was felt of interest not only to identify the species which had been isolated, but also to investigate their nutritional requirements.

The nutrition of the lactic acid bacteria which occur in milk has been studied by many workers (reviewed by Dunn, 1949; Knight, 1945), but apart from a communication by Rainbow (1952) which described the requirements of four strains of lactobacilli isolated from beer, the literature is devoid of references to the nutritional requirements of beer lactic acid bacteria.

METHODS

The basal medium used was essentially that of Dunn, Shankman, Camien & Block (1947), differing only in the concentration of adenine, guanine, xanthine and uracil which were all used at a concentration of 12 mg./l. In general, media were made up from stock solutions of each organic constituent as desired. Growth of the organisms was measured turbidimetrically against uninoculated control blanks, using a Hilger 'Spekker' absorptiometer with neutral grey filters (H508). Values (expressed as 1000 × drum reading) were the means of triplicate tubes. The tubes were test tubes 4 in. in length each containing 3 ml. medium. They were sterilized by autoclaving at 10 lb./sq.in. for 2·5 min. (Toennies & Gallant, 1948).

Organisms were maintained in stab cultures and were transferred at monthly intervals. Reserve cultures were kept in the presence of $CaCO_3$ in sealed tubes. Though other workers have found yeast-extract glucose broth to be a suitable inoculum medium for lactic acid bacteria, our organisms grew slowly or not at all in such a medium. A medium of the following composition proved satisfactory: 1% (w/v) yeast extract (Difco); 0·5% (w/v) peptone (Evans); 1% (w/v) glucose; 1·2% (w/v) sodium acetate; 0·5% (v/v) each of salt solutions A and B (Snell & Wright, 1941). The reaction was adjusted to pH 5·1–5·3, using Johnson's pH papers.

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The cultures for inoculum were grown in 4 ml. medium in centrifuge tubes. These tubes were modified by the intrusion of glass prongs into the lumen about 3 cm. from the mouth. A cotton-wool plug was supported by the platform thus provided and so was not forced down during centrifugation. The cells were centrifuged, washed twice with 0·9% (w/v) saline and finally resuspended in 15–20 ml. saline. Pasteur pipettes were used to transfer one drop (about 0·05 ml.) of this suspension to each culture tube. The tubes were incubated at 25° in closed vessels in an atmosphere containing 80–85% CO_2. This procedure was adopted because the organisms studied normally grow anaerobically in the presence of a high concentration of CO_2.

RESULTS

Individual cultures were incubated for the particular period (5–8 days) which had been found in preliminary experiments to be optimal for maximum growth in the complete medium. Some organisms (not dealt with in this paper) failed to grow after 14 days incubation, thus showing that the complete medium was not suitable for all lactic acid bacteria.

The vitamin requirements of the bacteria are shown in Table 1, in which the organisms are arranged in order of their fastidiousness and the vitamins in order of their relative effect. A vitamin was termed 'essential' when its omission caused growth to decrease to one-quarter of that achieved in the complete medium; a fall to only one-half the turbidity reached in the control medium was deemed indicative of a 'stimulatory' vitamin, in order to facilitate classification.

The maximum number of vitamins required by any organism was four; *Lactobacillus parvus* required pantothenic acid, nicotinic acid, riboflavin and thiamine. Twelve strains (representing four species) required only pantothenic acid; all the organisms studied required this vitamin. Nicotinic acid was essential for twenty-two organisms and stimulated four others. In contrast, *p*-aminobenzoic acid (*p*-AB) was required by only one organism, *L. pastorianus* strain W4, while choline, inositol, the B6 group, folic acid and biotin were apparently not required by any of the organisms tested under our conditions.

It was thought of interest to attempt to grow some of the organisms on media containing only their 'essential' vitamins in addition to amino acids, purines, uracil, glucose and salts. Vitamin-free casein (Difco 1%, w/v), supplemented with tryptophan and cysteine, was used in place of the mixture of amino acids; other constituents were present in the same concentration as in the basal medium.

Eighteen media were prepared according to the vitamin requirements of eighteen organisms. Only *L. pastorianus* HH4 grew as well in this 'essential vitamin' medium as in the complete medium of vitamin-free casein + all vitamins. Of the remaining organisms the growth of five fell to between one-quarter and one-half of that in the complete medium, and of twelve

Table 1. *Vitamin requirements*

Organism	Ca pantothenate	Nicotinic acid	Riboflavin	Thiamine	*p*-AB	B6
*Lactobacillus parvus**	E	E	E	E	N	N
L. buchneri CC3	E	E	E	N	N	N
L. leichmannii EE4	E	E	E	N	N	N
L. malefermentans†	E	E	E	N	N	N
L. pastorianus HH4	E	E	N	E	N	N
L. pastorianus W4	E	E	N	N	E	N
L. bifidus DD1	E	E	S	N	N	N
L. bifidus DD5	E	E	N	N	N	N
L. bifidus EE1	E	E	N	N	N	N
L. buchneri α	E	E	N	N	N	N
L. buchneri β	E	E	N	N	N	N
L. buchneri 1	E	E	S	N	N	N
L. frigidus HH5‡	E	E	N	N	N	N
L. pastorianus BB1	E	E	N	N	N	N
L. pastorianus BB4	E	E	S	N	N	S
L. pastorianus DD4	E	E	N	N	N	N
L. pastorianus W5	E	E	N	N	N	N
L. pastorianus 12	E	E	N	N	N	N
L. pastorianus 19	E	E	N	S	N	N
L. pastorianus 23	E	E	N	N	N	N
Pediococcus damnosus var. *salicinaceus* FF6	E	E	N	N	N	N
Streptococcus cremoris 15	E	E	N	N	N	N
Lactobacillus buchneri C	E	N	N	N	N	S
L. buchneri K	E	S	S	N	N	N
L. leichmannii AA1	E	N	N	N	N	N
L. leichmannii AA2	E	N	N	N	N	N
L. leichmannii AA3	E	N	N	N	N	N
L. leichmannii AA4	E	N	N	N	N	N
L. pastorianus CC4	E	N	N	N	N	N
L. pastorianus W1	E	S	N	N	N	N
L. pastorianus W10	E	S	N	N	N	N
L. pastorianus D	E	S	S	N	N	N
L. pastorianus L	E	N	N	N	N	N
L. plantarum φ	E	N	N	N	N	N

E, essential (turbidity below one-quarter of control); S, stimulatory (turbidity one-quarter to one-half of control); N, non-essential (turbidity above one-half control); remaining vitamins were all non-essential.

* Russell & Walker (1953*b*). † Russell & Walker (1953*a*). ‡ Bhandari & Walker (1953).

organisms to less than one-quarter of the latter. Thus, the vitamin requirements (shown in Table 1) are not the optimal requirements of seventeen of the eighteen organisms. The experiment was then repeated with the media modified by the addition of further vitamins. These were those considered most likely to improve growth; they were added singly and as a mixture.

Thiamine was the most potent vitamin in that six organisms (*L. buchneri β*, *L. pastorianus* strains 12, 19, 23 and DD4, and *Streptococcus cremoris* 15) grew very well in the presence of their essential vitamins + thiamine. It may not be mere coincidence that in all these cases the essential vitamins were nicotinic and pantothenic acids. In eight cases the addition of folic acid (but not *p*-AB) resulted in a significant increase in growth. Taken in conjunction with the lack of requirement for *p*-AB this would seem to indicate that the organisms can synthesize this vitamin at a rate sufficient to satisfy their needs, but that the synthesis of folic acid is a slower process. Even when all the vitamins found important when omitted singly were added as a group, three organisms still did not grow well. These were *Lactobacillus buchneri* 1, *L. pastorianus* CC4 and *L. plantarum* ϕ.

DISCUSSION

The finding that all the organisms required pantothenic acid is in accord with the work of Shankman, Camien, Block, Merrifield & Dunn (1947), and of Cheldelin, Hoag & Sarett (1945) with other lactic acid bacteria. Other work in this laboratory has shown that the components of pantothenic acid, supplied either separately or together, were not used in place of the intact vitamin.

Shankman *et al.* (1947) did not find *p*-AB essential for any of the twenty-three lactic acid bacteria they studied, but Isbell (1942) reported it to be essential for *L. arabinosus* 17-5, and Pennington (1946), found that it was required by *Leuconostoc mesenteroides* P-60 under certain conditions. It is to be noted that the requirement for *p*-AB observed in the present work was found on a medium which contained folic acid and purines. Evidently *Lactobacillus pastorianus* W 4 cannot utilize these substances in place of *p*-AB, unlike *L. pentosus* (Snell & Mitchell, 1942).

The lack of requirement for folic acid and biotin makes the brewery lactic acid bacteria rather different from the milk organisms. Of the latter, Snell (1948) listed nine which required biotin and three which required folic acid. Shankman *et al.* (1947) found two bacteria which were exacting towards biotin and four to folic acid, out of twenty-three organisms studied. The lack of a biotin requirement observed with our organisms may be linked to the presence of asparagine and a high concentration of CO_2, although oleate was absent.

In the absence of an 'essential' vitamin, growth generally dropped to much less than one-quarter of that in the complete medium. Frequently the decrease was to one-tenth, particularly when pantothenic and nicotinic acids were involved. Thus, successful assays for these vitamins might be developed with these organisms; especially is this indicated by the fact that the organisms were tested on a rich medium over fairly long incubation periods.

There were a number of cases of apparent inhibition of growth by vitamins. Thus, the omission of *p*-AB led to increased growth of eight organisms, and thiamin inhibited two strains. Shankman *et al.* (1947), stated that *p*-AB was inhibitory towards *L. casei*, while Whiteside-Carlson & Carlson (1949) found instances of inhibition of *Leuconostoc* sp. by riboflavin, biotin, folic acid, *p*-AB and vitamin B6.

Table 1 shows that the vitamin requirements of different strains of any one species varied greatly. Thus, with respect to *Lactobacillus buchneri*, strain CC3 required three vitamins, strains α and β required two and strains C and K each required only one vitamin. In the case of *L. pastorianus*, two strains had three requirements, seven had two requirements, and for five strains only one vitamin was necessary. This variation in nutritional requirements of strains of any particular species is well known (Boivin, 1945; Shankman *et al.* 1947). However, there appears to be a tendency for strains isolated from the same source to have the same requirements. Thus, strains AA1, AA2, AA3 and AA4 of *L. leichmannii* were all isolated from one yeast and all required only pantothenic acid; strain EE4, from a different yeast, required pantothenic acid, nicotinic acid and riboflavin. Strains α and β of *L. buchneri* isolated from one yeast both required pantothenic and nicotinic acids, whereas strains C and K, from another yeast, required only pantothenic acid. This trend to similarity is not shown by strains W1, W4 and W5 of *L. pastorianus* which were all isolated from one source but which required one, three and two vitamins, respectively.

Not many attempts appear to have been made to grow lactic acid bacteria on media which contained only their 'essential' vitamins. However, Whiteside-Carlson & Rosano (1951) found that *L. dextranicum* strain 'elai' did not grow on a basal medium + thiamine, nicotinic acid and pantothenic acid. The addition of a single vitamin was not effective in restoring growth completely, although folic acid gave a significant improvement.

Thanks are due to Dr F. Bergel, Roche Products Ltd., for the gift of pyridoxal acetal hydrochloride and pyridoxamine dihydrochloride. One of us (C.R.) was a Postgraduate Scholar in Technology of Manchester City Council and one of us (R.R.B.) held a Scholarship awarded by the Rajasthan Government.

This paper is the second of a series on lactic acid bacteria associated with brewery products.

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Exposure to Light as a source of Error in the Estimation of the Infectivity of Virus Suspensions

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SUMMARY: Experiments were made to determine whether the infectivity of virus suspensions was influenced by exposure to daylight of normal laboratory intensity or by exposure to artificial light. The infectivity of dilutions of virus suspensions held in bottles or cells was determined after exposure to light under a variety of conditions; the influence of exposure was indicated by comparison with the infectivity of control suspensions kept in darkness. The infectivity titres indicated by serial dilutions, in phosphate saline, of unfiltered fresh suspensions of egg strains of the viruses of vesicular stomatitis, influenza, Newcastle disease and fowl-plague following exposure to daylight for 4 hr. were 3–5 logarithmic units lower than those indicated by the dark controls. Changes of this magnitude suggest that with these virus strains significant errors might arise in infectivity titrations if suspensions were exposed to light for much shorter periods. Inactivation due to exposure to daylight was demonstrated in similar experiments with an egg strain of vaccinia virus, a mouse neurotropic strain of influenza virus and with guinea-pig strains and mouse neurotropic strains of the virus of vesicular stomatitis. Results of experiments with the virus of vesicular stomatitis indicated that inactivation by exposure to light may be eliminated almost completely by the inclusion of 10% rabbit serum in the suspending medium. Losses of infectivity increased with the intensity of illumination and with the duration of exposure. Filtration or storage of certain initial materials also increased the susceptibility to inactivation of virus suspensions derived from them. The infectivity of suspensions of the virus of foot-and-mouth disease, even after filtration, was relatively stable during exposure to light.

During the course of infectivity titrations designed to determine the regularity of response of chick embryos to the inoculation of fourfold dilutions of an egg strain (Ind. C) of vesicular stomatitis virus, it was observed on one occasion that the infection rate was unexpectedly low for the two lowest dilutions when their inoculation was delayed for 30–100 min. longer than usual. In two further titrations with the same egg strain dilutions containing originally 25 and 80 ID 50 were no longer infective when re-inoculated after an interval of 3 hr.

The standard procedure for infectivity titrations at that time (July 1947) was to prepare dilutions of virus suspensions in screw-capped bottles and to leave these on the laboratory bench whilst eggs were being prepared for inoculation of the chorioallantoic membranes. Groups of 6 eggs were inoculated with each dilution, beginning with the highest, the inoculation being made as each membrane was dropped. One egg was inoculated every minute, and at least 30 min. were required to complete the inoculation of a series of five dilutions.

In view of the demonstration of the instability of the infectivity of suspensions of this strain of virus a new procedure for infectivity titration was adopted thenceforth in which the eggs were prepared first. The virus suspension was then diluted serially and the dilutions inoculated immediately into the prepared eggs at the rate of about 6 per min. Using this rapid procedure it was found that the infectivity titre indicated by a series of dilutions was higher by more than one logarithmic unit than when inoculation was repeated after the same dilutions had been stored for 1 hr. on the laboratory bench at room temperature or in iced water. This difference was due to the complete loss of the infectivity of the highest dilutions. Similar results were obtained whether dilutions were prepared in Hartley's digest broth, saline, or phosphate saline (pH 7·6). Inactivation was slight, however, when dilutions were stored in the dark for prolonged periods.

It was concluded, therefore, that exposure to daylight was a major factor in the mechanism of the reduction of infectivity. It was evident that in many normal procedures for infectivity titrations the influence of exposure to light might remain undetected and could be the cause of serious errors if suitable precautions were not adopted. The subject of the present paper is a comparative study of the influence of exposure to light on the infectivity of suspensions of the viruses of vesicular stomatitis and some other diseases.

METHODS

Virus strains and test animals employed

Foot-and-mouth disease virus. Guinea-pig adapted virus of the following strains (immunological types) was used: strain 1 (Vallée O); ASJ (Vallée O); GB (Vallée A); GC (Waldmann C); Bec 1 (S.A.T. 1); Rho 1 (S.A.T. 2). Guinea-pigs (420–600 g.) were used as experimental animals and were inoculated by intradermal tracking of the metatarsal pads. Nagel's neurotropic mouse strain, type Vallée A (Nagel, 1937; Hofmann, 1941, 1944) was also used.

Vesicular stomatitis virus. Virus of both immunological types was used, the Ind. C and Mex 1 strains of the Indiana type and the NJ. M strain of the New Jersey type. The infectivity of virus from bovine tissue was tested on chick embryos. In most of the experiments virus strains were used which had been passaged in guinea-pigs, mice or chick embryos (7–10 days old) by the intradermal, intracerebral and chorioallantoic methods respectively. These same hosts were used in infectivity tests.

Influenza, Newcastle disease and fowl plague viruses. The PR 8 strain of influenza A virus, the Lee strain of influenza B virus, and the strains of Newcastle disease and fowl plague had been propagated by the allantoic method in 9- to 10-day old chick embryos. The same host and methods of inoculation were used in infectivity tests. Infection in embryos inoculated with influenza virus was determined by a fowl cell haemagglutination test of allantoic fluid collected 2–3 days after inoculation. This test was also made in experiments with the two other viruses to check the specificity of deaths of embryos. The W.S. neurotropic mouse strain of influenza A was also used.

Vaccinia virus. The virus was an egg-adapted strain and chick embryos aged 11–12 days were inoculated by the chorioallantoic method.

Preparation of virus suspensions

Unless otherwise stated, virus suspensions were prepared on the day of the experiment from infective tissue freshly collected or stored for not more than a week at 4°. The suspending medium was 0·15 M-NaCl adjusted to pH 7·6 by the addition of phosphate salts. This medium is called 'phosphate saline' and the molarity of the phosphate salts is indicated in the text. Serum, when added to the medium, was first heated to 56° for 30 min. The amount of serum added is indicated as % (v/v).

Suspensions were prepared by grinding infective tissue in the proportion of 1 g. to 9 or 24 ml. of suspending medium. After clarification by centrifugation the supernatant was taken as the initial virus suspension or was first filtered by negative pressure through an asbestos pad of 'Seitz K' or 'Seitz EK' porosity. Such supernatants or filtrates are referred to as 1/10 or 1/25 tissue extracts. Gradocol membranes of 0·6 μ. A.P.D. were also used to filter egg fluids or Seitz K filtrates of tissue suspensions.

All manipulations were performed on a bench in front of windows facing west. On bright days window blinds were drawn. Filter flasks were covered with a thick cloth and all glassware containing virus suspensions was kept in the dark. The light intensity to which suspensions were exposed during pipetting operations was normally about 300 ft.c. and probably never exceeded 600 ft.c.

Methods of exposure of virus suspensions to daylight

Method of single dilutions. In this method a single dilution of the initial virus suspension was distributed as two 5 ml. samples in 25 ml. screw-capped bottles (McCartney pattern). One of these bottles was exposed to daylight on the laboratory bench and the other kept in the dark at the same temperature to serve as a control. The exposed bottle was immersed to a depth of about 1 in. in water in a shallow white enamelled tray, the water being maintained at room temperature or at 4°. At the conclusion of the period of exposure both bottles were placed in the dark at 4° and were taken at once to the inoculation room where the exposed sample and then the control sample were titrated for infectivity. All dilutions were made in the same medium as that used to prepare the exposed virus suspension. Any significant difference between the infectivity titres of the two samples was an indication of the error which could arise in infectivity titrations if virus suspensions were exposed to light before the preparation of dilutions.

The standard procedure for titration was to prepare in 25 ml. screw-capped bottles tenfold dilutions of the starting material by carrying over 0·5 ml. into 4·5 ml. and mixing by gently shaking for about 5 sec. In all cases the starting material was defined as the 10^0 dilution. A new pipette was used for each dilution and the bottles were placed at once in the dark. The set of dilutions was inoculated immediately after preparation beginning with the highest dilution, the required animals or eggs having been prepared previously. In intradermal titrations in the guinea-pig multiple tracks on both metatarsal pads were inoculated liberally. The standard volume inoculated into eggs was

0·1 ml. and into mice 0·03 ml. Undue exposure to light of the glass inoculating syringe was avoided. From the accumulated positive and negative observations at each dilution the 50% positive end-point dilution was calculated by the method of Reed & Muench (1938).

Method of multiple dilutions. In this method tenfold serial dilutions of the initial virus suspension were distributed in two identical series as 5 ml. samples in 25 ml. screw-capped bottles. One series was exposed to light and the other kept in the dark as described for the method of single dilutions. At the conclusion of the period of exposure the two series of dilutions were placed in the dark at 4° and were at once tested, without further dilution, by inoculation into groups of the appropriate host. The exposed series was always inoculated first. A 50% positive end-point dilution was calculated from the host response as though the dilutions were those of a standard infectivity titration. The method of multiple dilutions was essentially an infectivity titration in which a delay occurred between preparation and inoculation of the dilutions. Exposure periods of up to 4 hr. were employed and any significant difference between the two end-point dilutions indicated inactivation due to light.

Methods of exposure of virus suspensions to artificial light

In view of the variable intensity of daylight it was essential, when comparison was to be made between different experiments, to employ a source of light of constant intensity. For this purpose the apparatus shown in Pl. 1 was employed. A 250 W. compact-source mercury vapour lamp fitted with a glass window (Type M.E., obtained from the General Electric Co. Ltd.) was mounted in a sliding holder at the focus of a 10 cm. condenser lens. A specially constructed glass exposure cell was supported centrally in the parallel beam of light at a distance of 40 cm. from the arc. The exposure cell, which is shown to scale in horizontal section in Fig. 1, was constructed of 4 mm. thick plates of hard glass. Each of the five cell compartments was 1 cm. wide, 5 cm. deep and 1·5 cm. long in the direction of incident illumination. All surfaces of the cell not normal to the direction of incident illumination were ground to a smooth translucent finish.

The intensity of incident illumination was 3800 ft.c. and was constant to within 5% over the entire face of the cell. In some experiments the condenser lens was omitted and the position of the exposure cell was adjusted to provide an intensity of incident illumination of between 770 and 3000 ft.c. In other experiments samples of a single virus suspension were exposed simultaneously to different intensities of illumination by the attachment to the face of the cell of neutral density filters of known light transmission. This arrangement is shown in Fig. 1A. Experimental samples in the central and outside compartments of the cell were thus exposed to 100, 16 and 1% of the intensity of incident illumination. Scattering of light within the cell was minimized by filling the intermediate compartments with an optically dense solution of potassium permanganate. The apparatus was set up in a darkened room or cold store in order to maintain the temperatures of exposed samples at 20° or 4° respectively. Heating effects due to radiation from the lamp were controlled by a blast of cold air directed at the face of the cell. The temperature of the exposure cell was indicated by a thermometer placed in saline in one of the compartments. The cell compartments were filled with samples of about 5 ml. and were sealed with glass cover-slips. In all cases identical control samples were held in the dark at the same temperature. At the conclusion of the period of exposure all samples were removed to 25 ml. McCartney bottles and held in the dark in iced water until required for infectivity titration. This standard procedure was equivalent to the method of single dilutions described above.

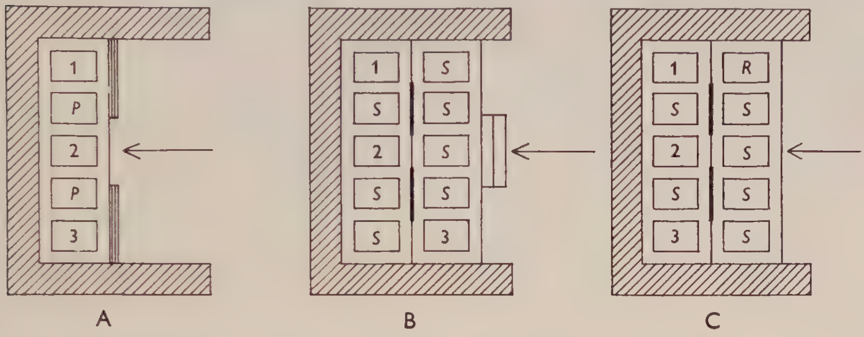


Fig. 1. Schematic horizontal sections showing arrangements of exposure cells in collimated beam of artificial light. The arrows indicate the direction of the incident illumination. A, arrangement of a single cell for Expts. 7A–C: neutral density filters attached to the face of the cell. Samples of a single virus suspension exposed to 100 % of the incident intensity in the central compartment (2) and to 1 and 16 % of the incident intensity in the outside compartments (1 and 3). Intermediate compartments (*P*) contain an optically dense solution of potassium permanganate. B, arrangement of two cells for Expt. 7E: sample of a single virus suspension exposed in compartment 3 to an incident intensity of 3800 ft.c. and in compartments 1 and 2 to intensities of 3100 and 2400 ft.c. obtained by the insertion into the beam of two and four additional thicknesses of glass respectively. Intermediate compartments are screened by opaque paper placed between the cells. The compartments *S* contain phosphate saline. C, arrangement of two cells for Expt. 7G: virus suspensions exposed with (compartment 3) and without (compartments 1 and 2) the addition of 10 % rabbit serum to the suspending medium. Incident illumination modified by transmission through compartments filled with phosphate saline (*S*) or phosphate saline containing 10 % rabbit serum (*R*). Intermediate compartments are screened by opaque paper placed between the cells.

In Expts. B, D and E (Table 5), in order to employ the method of multiple dilutions, the cell was not used and the dilutions of virus suspensions were exposed in 5 ml. McCartney bottles arranged on a platform in the uncollimated beam of light.

Measurement of incident light intensity

Daylight. In the earliest experiments frequent meteorological observations were made and the average relative intensity of incident light was estimated by reference to photographic exposure tables. This scale of relative values was converted later to an absolute scale by calibration with the S.E.I. photometer (manufactured by Salford Electrical Instruments, Ltd. and distributed by Ilford Limited, Ilford, London). Such converted values are indicated in the text as approximate. Absolute determinations of incident light intensity were made by means of a calibrated photoelectric circuit or the S.E.I. photometer the latter instrument being the ultimate standard. When the S.E.I. photometer was employed the intensity of incident illumination was derived from the observed brightness of a standard reflecting surface placed in the required position. A sheet of clean white filter-paper of 85% reflectance was used as a convenient standard surface.

Light intensity at the laboratory bench decreased rapidly with distance from the windows. Approximate average readings obtained during the summer months (April–August) 30 in. from windows facing west, for the period 10 a.m. to 2 p.m., were 400 ft.c. when the sky was cloudless, and 180/300/700 ft.c. when the sky was completely overcast with low/medium/high cloud. With breaks in the cloud the intensity increased considerably and with cumulus cloud the intensity occasionally approached that of direct sunlight at about 3000 ft.c. These intensities were decreased by about 20% in the spring and autumn and by about 40% in the winter.

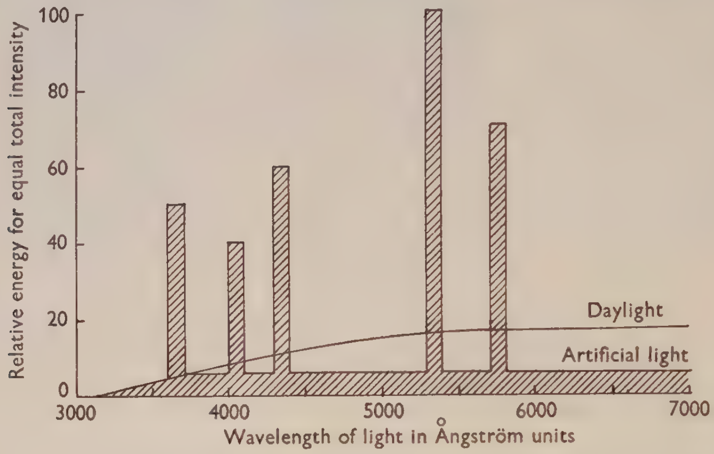


Fig. 2. A schematic representation of the spectral distributions of daylight and the light of the artificial source (shaded).

Artificial light. The intensity of illumination produced by the artificial source was measured either with a calibrated photoelectric circuit or with the S.E.I. photometer as described above.

Comparison of the spectral distributions of daylight and the light of the artificial source. Under the conditions defined above the artificial source produced at the exposure cell an intensity of illumination which was approximately equivalent to that of direct sunlight. The spectral qualities of the two sources, were, however, quite different. It is shown in Fig. 2 that the unmodified light from the artificial source consisted of the intense lines of the mercury spectrum superimposed upon a background of much lower intensity: the spectral distribution of sunlight is continuous. The significantly greater energy of wavelengths shorter than 4000 A. in the artificial light was compensated, however, by the association of the artificial source with a glass lens of about 1 cm. mean thickness and a cell wall of glass 4 mm. thick. These thicknesses were such that the experimental sample contained by the exposure cell received only about a quarter of the energy of the 3600 A. line and no energy of wavelength shorter than 3100 A. A similar cut-off at 3100 A. applied in the experiments in which virus suspensions were exposed to daylight transmitted by window glass and the wall of the containing bottle. It is reasonable, therefore, to conclude that any change observed following the exposure of an experimental sample to the light of the artificial source was associated with energy of those wavelengths which are present in daylight.

Presentation of results

For convenience of presentation infectivity titres are expressed as the logarithms to the base 10 of the reciprocals of the 50 % end-point dilutions. Thus a 50 % end-point dilution of $10^{-3.0}$ is expressed as an infectivity titre of 3·0. An infectivity titre of <3.0 indicates an end-point dilution between 10^{0} and $10^{-3.0}$. Examples of the number of animals or eggs inoculated with each dilution are given in Table 4. In all experiments the number of observations made at each dilution were such that a change of infectivity titre of one logarithmic unit may be regarded as significant. The number appearing in reference to any experiment indicates the number of the Table in which the results are presented.

RESULTS

Influence of storage in the dark on the infectivity of virus suspensions

In all of the present experiments there was an inevitable delay between the termination of exposure to light and the beginning of inoculation of test animals. During this short period the bottles containing the virus suspensions were immersed in iced water in the dark. The influence of this procedure on the infectivity of suspensions of the Ind. C and NJ. M guinea-pig strains of vesicular stomatitis virus was determined in the following two experiments. In each experiment three fivefold dilutions, including the estimated 50 % end-point dilution, of an unfiltered extract of guinea-pig vesicular pad epithelium were prepared in 0·005 M-phosphate saline and were distributed in two identical sets as 10 ml. samples in 25 ml. screw-capped bottles. One set was placed at once in the dark in iced water in a closed metal container, and after 15 min. was inoculated intradermally into guinea-pigs; eight animals were used at each dilution. The dilution bottles were returned to the metal containers immediately after the inoculation samples had been withdrawn. This procedure was repeated twice with the same dilutions at 15 min. intervals. The second set of dilutions was inoculated twice into guinea-pigs in a similar manner after storage for 4·25 and 4·5 hr. in the dark at 4°.

The results are summarized in Table 1 and indicate that during storage for 15–45 min. in the dark at 4° inactivation of the virus was not detected. Confidence was thus established in the routine procedure of storage of virus suspensions whilst awaiting inoculation. Over the longer periods of 4·25 and 4·5 hr. the only significant inactivation observed was that of the highest dilution in the experiment with the Ind. C strain.

Table 1. *Virus of vesicular stomatitis. Influence of storage in the dark at 4° on the infectivity of minimal infective dilutions prepared in* 0·005 M-*phosphate saline*

		Period of storage (hr.)				
		0·25	0·5	0·75	4·25	4·5
Strain	Dilution of 1/25 unfiltered extract of guinea-pig pad epithelium	Number of positive reactions at sixteen inoculated sites on eight guinea-pigs				
Ind. C	$10^{-3 \cdot 5}$	9	7	9	4	0
	$10^{-2 \cdot 8}$	14	16	15	15	13
	$10^{-2 \cdot 1}$	16	16	16	16	15
NJ. M	$10^{-3 \cdot 8}$	2	0	0	1	2
	$10^{-3 \cdot 1}$	4	4	5	3	2
	$10^{-2 \cdot 4}$	14	10	12	13	8

In five similar experiments (3A, 3B, 3C, 5C and 5D) with the guinea-pig strains of vesicular stomatitis virus sets of dilutions were tested after 30 min. and 4 hr. storage in the dark at 4° or 20°. The indicated infectivity titres fell during the intervening period by 0·4–0·6. In four experiments (3C, 3D, 3E and 3F) performed under identical conditions with guinea-pig strains of foot-and-mouth disease virus there was a fall in titre of 0·4 in one experiment but no fall in the other three. These titres were based on only six to eight observations at each tenfold dilution. Individually none of these changes was therefore significant, but the consistency of the decreases in titre in experiments with the virus of vesicular stomatitis again suggested that over a period of 4 hr. in the dark minimal infective dilutions may be inactivated.

It is well to emphasize that in the experiments which form the main part of this paper the inactivation of virus suspensions due to exposure to light was estimated from the differences of infectivity between identical suspensions exposed to light and kept in the dark at the same temperature and for the same period. Thus, apart from the experiments with the viruses of vesicular stomatitis and foot-and-mouth disease discussed above, no data have been obtained on the concurrent inactivation which may occur in control suspensions during storage in the dark.

Influence of exposure to daylight on the infectivity of virus suspensions

Experiments with different strains of the virus of vesicular stomatitis obtained from various sources. Before the methods of single and multiple dilutions had been established as routine procedures three experiments were made with the Ind. C egg strain of vesicular stomatitis virus in which titrations of unfiltered 1/10 extracts of chorioallantoic membrane indicated infectivity titres of 6·8–7·5 when dilutions were prepared in 0·005 M-phosphate saline and inoculated immediately. The titres indicated by the same dilutions after leaving them for 1–1·5 hr. in daylight at room temperature were 1·5–1·8 units lower. In one of these experiments a duplicate set of dilutions kept in the dark as a control indicated that the fall in titre due solely to exposure to light was 1·0 unit. The results of experiments with three other sources of this virus are detailed in Table 2, and in each instance significant decreases in infectivity titres were observed after exposure to daylight.

Table 2. *Virus of vesicular stomatitis. Influence of exposure to daylight on the infectivity titre of tissue extracts. Exposure by the method of single dilutions at 4°*

Strain	Infective tissue	Extract exposed	Average light intensity (ft.c.)	Period of exposure (hr.)	Infectivity titre		Decrease of titre due to exposure
					Control	Exposed	
Ind. C.	Mouse brain	1/10,000 in 0·005 M-phosphate saline, unfiltered	~1800	3·5	2·2	Inactive	⩾2·7
Mex. 1	Bovine tongue epithelium	1/25 in Hartley's digest broth filtered 0·6 μ. A.P.D. membrane	~ 400	6	5·1	2·8	2·3
NJ. M	Guinea-pig pad epithelium	1/25 in 0·005 M-phosphate saline filtered 0·6 μ. A.P.D. membrane	~ 240	6	4·4	1·1	3·3

Comparative experiments with the viruses of vesicular stomatitis and foot-and-mouth disease. In the standard procedures employed at this institute for the infectivity titration of foot-and-mouth disease virus in guinea-pigs no special precautions are taken to eliminate exposure of the virus suspensions to indirect sunlight. The incident light intensity is decreased considerably, however, by the racks in which the sets of dilutions are held. In hot weather these racks are immersed in iced water in an open metal container. All sets of dilutions are prepared in the laboratory before taking them to the animal units for inoculation. Protective rubber clothing is always worn in these units and the time taken to change involves a delay of at least 10 min. between completion of the manipulations in the laboratory and the beginning of inoculation. When comparative titrations of twelve samples are undertaken at one time the necessary manipulations involve a delay between preparation and inoculation of each series of dilutions which increases from 1 hr. for the first series to 2 hr. for the twelfth. There has been no indication that exposure to daylight for up to 2 hr. under these conditions has any significant influence on the infectivity titre of suspensions of the virus of foot-and-mouth disease. This same standard procedure was adopted with guinea-pig strains of vesicular stomatitis virus until dilutions of egg strains of this virus were observed to be unstable unless kept in the dark. A comparison of the stability of guinea-pig strains of the viruses of vesicular stomatitis and foot-and-mouth disease was then made in experiments which are summarized in Table 3. The inoculation of four identical sets of dilutions was delayed by storage in the dark for 30 min. and for 4 hr. at 4°, and in daylight for 4 hr. at 4° and at 20°. The changes which occurred in the control suspensions stored in the dark have already been discussed.

In only one of the four experiments with foot-and-mouth disease virus was there a significant decrease of infectivity titre caused by exposure to daylight.

In this experiment (3D) the estimated intensity of illumination was the greatest of the series and included several bright periods. In experiment 3C, where the two viruses were exposed simultaneously, the greater stability of the virus of foot-and-mouth disease was demonstrated clearly. It is apparent that in the routine titration procedure adopted with foot-and-mouth disease virus exposure to daylight is without significant influence on the infectivity titre even if all manipulations are carried out at 20°.

Table 3. *Viruses of vesicular stomatitis and foot-and-mouth disease. Influence of exposure to daylight on the infectivity of dilutions of unfiltered extracts of guinea-pig pad epithelium prepared in* 0·04 M-*phosphate saline. Exposure by the method of multiple dilutions at* 4° *and* 20°

Expt.	Virus	Strain	Average light intensity (ft.c.)	Period of exposure (hr.)	Infectivity titre indicated by			Decrease of titre due to exposure at 4°
					Control dilutions	Exposed dilutions		
					4°	4°	20°	
A	Vesicular stomatitis	NJ. M	~300	0·5	4·9			
				4	4·4	⩽2·1	⩽2·1	⩾2·3
B		NJ. M	~400	0·5	4·6			
				4	4·2	3·0	2·2	1·2
Ci		Ind. C	~240	0·5	4·6			
				4	4·2	2·2	1·2	2·0
Cii	Foot-and-mouth disease	GC	~240	0·5	4·6			
				4	4·8	4·2	4·5	0·6
D		GB	~600	0·5	4·3			
				4	4·5	3·3	3·4	1·2
E		1	~240	0·5	4·6			
				4	4·6	4·6	4·4	0·0
F		1	~180	0·5	4·3			
				4	3·9	3·6	3·9	0·3

In the three experiments with vesicular stomatitis virus the 4 hr. period of exposure to daylight at 4° caused decreases in the indicated infectivity titres from 1·2 to ⩾2·3 units. In Expts. 3B and 3C the titres indicated by the dilutions exposed at 20° were about 1·0 unit lower than those indicated by the dilutions exposed at 4°, but there were no dark controls at the higher temperature. In Expt. 3A the sets of dilutions stored in the dark for 4 hr. at 4° and exposed to daylight for 4 hr. at 20° were also tested by inoculation of chick embryos and the respective titres were ⩾5·1 and ⩽2·1.

In two similar experiments (5A and 5C) with the guinea-pig strains of the virus of vesicular stomatitis the dilutions were prepared from filtrates and were exposed at 20° only. Infectivity titres were reduced by at least 2·0 units following exposure for 4 hr. In Expt. 5C the indication was that on bright days an exposure period of only 1 hr. might produce a significant reduction of infectivity titre. With this virus the phenomenon of inactivation by exposure to light could clearly lead to false interpretations unless appropriate precautions were taken.

Table 4. *Influence of exposure to daylight on the infectivity of dilutions of unfiltered egg fluids and tissue extracts prepared in* 0·005 M-*phosphate saline. Exposure by the method of multiple dilutions at 4° for 4 hr.*

Expt.	Source of infective tissue	Test animal and number inoculated with each dilution	Average light intensity (ft.c.)	Virus and strain	Infectivity titre indicated by		Decrease of titre due to exposure
					Control dilutions	Exposed dilutions	
A	Chick embryo allantoic fluid	Chick embryo, 8	240	Influenza B, Lee	8·2	⩽4·5	⩾3·7
				Newcastle disease	8·5	5·8	2·7
B	Chick embryo allantoic fluid	Chick embryo, 8	200	Newcastle disease	8·5	7·3	1·2
				Fowl plague	8·8	5·7	3·1
C	1/10 extract of chick embryo chorioallantoic membrane	Chick embryo, 6–7	350	Vesicular stomatitis, Ind. C	6·8	⩽1·9	⩾4·9
				Vaccinia	5·9	4·3	1·6
D	1/10 extract of mouse brain	Mouse, 6–8	390	Foot-and-mouth disease, Nagel	4·4	4·5	−0·1
				Influenza A. W.S. neurotropic	3·4	2·1	1·3
E	1/10 extract of mouse brain	Mouse, 8	410	Vesicular stomatitis, Ind. C	4·4	2·5	1·9
				Vesicular stomatitis, NJ. M	4·0	2·5	1·5
F	Extract used in Expt. E after storage for 11 days at 4°	Mouse, 8	350	Vesicular stomatitis, Ind. C	4·0	1·5	2·5
				Vesicular stomatitis, NJ. M	2·4	1·3	1·1

Experiments with the viruses of influenza, Newcastle disease, fowl plague and vaccinia. Table 4 contains the results of experiments in which several viruses were exposed to daylight at 4° by the method of multiple dilutions. A valid comparison of two viruses was made in each experiment by using identical sources of infective tissue and suspending medium. A significant inactivation due to exposure to daylight occurred with egg strains of the viruses of influenza B, Newcastle disease, fowl plague and vaccinia. In the experiment comparing neurotropic strains of the viruses of foot-and-mouth disease and influenza A significant inactivation occurred only with the latter virus and to a degree comparable with that observed with the neurotropic strains of the virus of vesicular stomatitis.

Influence of exposure to artificial light on the infectivity of virus suspensions

Experiments with guinea-pig strains of the virus of vesicular stomatitis

(*a*) *Comparison of daylight and artificial light.* In two experiments (5C and 5D) made on consecutive days the decrease of the infectivity of virus suspensions caused by exposure to daylight was compared with that caused by artificial light of the same intensity. Portions of epithelium from the same guinea-pig pads were used in both experiments and the incident intensity of artificial light in Expt. 5D was adjusted to correspond with that of the daylight in Expt. 5C. The results indicated that within the limits of significance

Table 5. *Virus of vesicular stomatitis. Influence of exposure to daylight or artificial light on the infectivity of dilutions of filtered* (0·6 μ.) 1/25 *extracts of guinea-pig pad epithelium prepared in* 0·04 M-*phosphate saline. Exposure in bottles by the method of multiple dilutions at* 20°

					Infectivity titre indicated by		
Expt.	Virus strain	Source of light	Light intensity (ft.c.)	Period of exposure (hr.)	Control dilutions	Exposed dilutions	Decrease of titre due to exposure
A	NJ. M	Daylight	780	4	2·6	⩽0·1	⩾2·5
B	NJ. M	Artificial	2700	2		⩽0·5	⩾1·7
				4	2·2	⩽0·5	
C	Ind. C	Daylight	1300	0·5	2·6	2·0	0·6
				1		1·6	
				2		1·1	
				4	2·1	⩽0·1	⩾2·0
D	Ind. C	Artificial	1300	0·5	3·0	2·1	0·9
				1		1·1	
				2		1·0	
				4	2·4	⩽0·1	⩾2·3
E	Ind. C	Artificial	2700	0·5	2·9	⩽1·6	⩾1·3

In Expts. A, B and E duplicate sets of dilutions prepared with 1 % guinea-pig serum added to the diluent were exposed simultaneously. In Expts. B and E there were also sets with 5 % guinea-pig serum added. In all cases the control dilutions indicated an infectivity titre which varied by only 0·2 unit or less from those shown above for the dilutions with no serum added. All the exposed dilutions were inactivated to the same extent as those with no serum added with the single exception of those with 5 % serum in Expt. E which indicated a titre of 2·0.

equal intensities of illumination by either source produced the same decreases in infectivity. A similar comparison of the two sources of light was made in Expt. 6D. The decrease of the infectivity titre was again of the same magnitude in both cases.

(*b*) *Filtration as a factor influencing susceptibility to inactivation.* In five experiments (6D–6H) virus suspensions were prepared from unfiltered extracts of guinea-pig pad epithelium and were exposed to light at 4°. The decrease of infectivity was less than might have been anticipated from the results of other experiments (5B, 5D, 5E and 6A) in which suspensions were prepared from filtered extracts and were exposed to light at 19–20°. This suggested that inactivation due to exposure to light was influenced either by the filtration of the initial extract or by the temperature. Results with other strains of this virus indicated that filtration was probably the major factor. If the effect produced by filtration were due only to the removal of particulate matter greater than a certain size then it should be possible to produce the same effect by an equivalent centrifugation procedure. An investigation was made of the influence of such a procedure and of the molarity of the phosphate salts.

A 1/6·25 extract of guinea-pig pad epithelium infected with the NJ. M strain of virus was prepared in 0·04 M-phosphate saline. By further dilution in 0·04 M-phosphate saline, and in saline alone, two 1/50 extracts were prepared. These were termed the 0·04 M- and the 0·005 M-phosphate saline 'low-speed supernatants' respectively. A 50 ml. sample of the 0·005 M-phosphate saline low-speed supernatant was clarified further by high-speed centrifugation sufficient to remove particles of a diameter greater than about 0·1 μ. From this supernatant, a sample of 35 ml. was withdrawn and termed the 'high-speed supernatant'. A 14 ml. sample of the high-speed supernatant was filtered through a 0·6 μ. A.P.D. gradocol membrane (4 cm. diameter) which, in the absence of an adsorption effect, would remove no further particles from the high-speed supernatant. A similar sample was also filtered through a Seitz EK pad (3 cm. diameter). Each of the five suspensions obtained in this way was diluted once in its own buffer to give a 1/100 suspension which was exposed in the cell for 4 hr. at 4° to a light intensity of 3800 ft.c. The suspensions were added to the cell as they became available and in this way it was possible to titrate all exposed samples and the dark controls immediately after the period of exposure. The results are given below:

	Infectivity titre		Decrease in titre due to
Suspensions	Control	Exposed	exposure
0·04 M-phosphate saline low-speed supernatant	3·5	2·6	0·9
0·005 M-phosphate saline low-speed supernatant	4·0	2·9	1·1
0·005 M-phosphate saline high-speed supernatant	3·5	2·4	1·1
High-speed supernatant filtered 0·6 μ membrane	3·4	1·3	2·1
High-speed supernatant filtered Seitz EK pad	Inactive	Inactive	—

The non-infectivity of the Seitz EK filtrate was not unexpected in view of the absence of 50 % Hartley's digest broth from the suspending medium. The decrease of the infectivity titres was the same in the three unfiltered suspensions but was significantly greater in the suspension prepared from the Gradocol

Table 6. *Viruses of vesicular stomatitis (VS) and foot-and-mouth disease (FMD). Influence of exposure in the cell to artificial light on the infectivity of suspensions prepared from guinea-pig vesicular fluid or extracts of pad epithelium*

Expt.	Initial material from which dilutions were prepared in the same suspending medium for exposure to light	Dilution exposed	Temperature of sample (°)	Light intensity (ft.c.)	Period of exposure (hr.)	Virus and strain	Infectivity titre		Decrease of titre due to exposure
							Control	Exposed	
A	1/60 pad extract in Hartley's digest broth, filtered Seitz K	Undiluted	19	770	4	VS, Ind. C	3·3	0·8	2·5
	1/60 pad extract in 0·005 M-phosphate saline, filtered Seitz K	Undiluted				VS, Ind. C	2·6	0·3	2·3
B	1/60 pad extract in 0·005 M-phosphate saline, filtered Seitz K	Undiluted	16	770	4	FMD, GB	3·7	3·7	0·0
C	1/32 pad extract in 0·005 M-phosphate saline, filtered Seitz K	1 in 1000	14	770	4	FMD, GB	1·4	0·5	0·9
D*	1/25 pad extract in 0·04 M-phosphate saline, unfiltered	1 in 4	4	770	4	VS, Ind. C	3·3	2·5	0·8
						FMD, Rho 1	4·1	3·7	0·4
E	As in Expt. D	1 in 40	4	1500	4	VS, Ind. C	2·0	0·4	1·6
						FMD, Bec 1	1·9	2·1	−0·2
F	As in Expt. D. Dilutions also prepared for exposure with guinea-pig serum added	1 in 400, no serum	4	1500	4	VS, Ind. C	0·5	Inactive	≥1·0
		1 in 400, 0·1 % serum				VS, Ind. C	0·8	0·0	0·8
		1 in 400, 1·0 % serum				VS, Ind. C	1·3	0·9	0·4
G	1/25 pad extract in 0·005 M-phosphate saline, unfiltered	1 in 12	4	3800	2	VS, NJ. M	2·0	1·9	0·1
H	As in Expt. G	1 in 4	4	3800	4	VS, NJ. M	2·8	1·6	1·2
					2	VS, Ind. C	2·5	2·5	0·0
					4	VS, Ind. C	2·4	1·5	0·9
I	1/25 pad extract in 0·005 M-phosphate saline, filtered Seitz EK	1 in 320	4	3800	4	FMD, GB	2·6	1·9	0·7
J	As in Expt. I	1 in 320	4	3800	4	FMD, GC	2·8	2·3	0·5
K	As in Expt. I	1 in 800	4	3800	4	FMD, Rho 1	2·9	2·4	0·5
L	As in Expt. I	1 in 100	4	3800	4	FMD, Bec 1	2·0	1·9	0·1
M	Partially purified, but unconcentrated, virus suspension obtained by one cycle of centrifugation from a 1/10 dilution of vesicular fluid in 0·067 M-phosphate buffer	Undiluted	20	3800	4	FMD, ASJ	5·0	4·7	0·3

* In Expt. D samples of the same suspensions as those exposed to artificial light were exposed simultaneously in an identical manner to daylight of the same intensity. The infectivity titres of these suspensions were 2·1 and 3·6 for the suspensions of the viruses of vesicular stomatitis and foot-and-mouth disease respectively.

membrane filtrate of the high-speed supernatant. These results suggest that in the course of filtration an adsorption process is largely responsible for the removal of a protective agent.

Experiments with guinea-pig strains of the virus of foot-and-mouth disease

To compare the susceptibilities of the viruses of foot-and-mouth disease and vesicular stomatitis suspensions were exposed under identical conditions in separate experiments (6A, 6B and 6C) or simultaneously in the same experiment (6D and 6E). To provide as severe a test as possible in further experiments (6I–6M) with the virus of foot-and-mouth disease the initial extracts were either filtered through a Seitz EK pad or purified by ultra-centrifugation. Suspensions were then exposed to the maximum intensity of illumination for 4 hr. For the titrations in Expts. 6I–6L five-fold dilutions were prepared and ten guinea-pigs were inoculated with each dilution. Under these conditions the observed decreases of infectivity titre of 0·5 and 0·7 unit may be regarded as significant. Greater decreases than these were always observed with suspensions of the virus of vesicular stomatitis prepared from extracts of guinea-pig pad epithelium even when these extracts were unfiltered and were exposed in lower dilutions under the same or less severe conditions. In Expt. 6M, in which extraneous proteins were largely removed by a single cycle of ultracentrifugation, the resuspended deposit suffered no significant loss of infectivity on exposure to light. In another experiment a similarly purified suspension obtained by two cycles of ultracentrifugation was exposed to a daylight intensity of 350 ft.c. for 4 hr. at 4° and the infectivity titres of the control and exposed samples were 5·3 and 5·1 respectively. These data confirm that the virus of foot-and-mouth disease is relatively resistant to inactivation by exposure to light.

Experiments with egg strains of the viruses of Newcastle disease and influenza

A pool of unfiltered amniotic fluid from 9-day-old chick embryos infected with the virus of Newcastle disease was used, after storage for 4 weeks at 4°, in an experiment to compare two suspending media; (i) 0·005 M-phosphate saline; (ii) a filtrate (0·6 μ) of normal amniotic fluid collected from 10-day-old chick embryos. Equivalent 1/3200 suspensions in each medium were exposed simultaneously for 4 hr. at 4° to an incident illumination of 3800 ft.c. The decrease of titre of $\geqslant 3{\cdot}2$ and $\geqslant 2{\cdot}8$ units, respectively, revealed no difference between the natural medium and the phosphate saline as suitable diluents for the virus. The same material was used 2 weeks later in Expt. 7C and a similar result was obtained.

In experiments with the viruses of influenza the suspending medium was 0·005 M-phosphate saline. Single dilutions prepared from unfiltered allantoic fluid (Lee strain) after storage for 2–4 weeks at 4° were exposed at 4° to 3800 ft.c. Dilutions of 1/100, 1/50,000 and 1/100,000 were exposed for 30 min.; the infectivity titres were decreased by 1·0, 0·8 and 1·0 units, respectively. In a similar experiment with the PR 8 strain a 1/10,000 dilution fell in titre by 1·9 units after exposure for 1 hr. A greater degree of inactivation was observed in two experiments with the Lee strain when a single filtrate (0·6 μ. A.P.D. membrane) of allantoic fluid was used after storage for 2 and 6 weeks. In these experiments samples of a 1/10,000 dilution were exposed simultaneously at 4° to 3800 ft.c. and 600 ft.c. in a cell with a neutral density filter attached. The suspension exposed to the higher intensity of illumination was removed after x min. and then stored for $5x$ min. in the dark. Exposure of the second suspension to one-sixth of this intensity was continued for a total of $6x$ min. to make the product of the intensity and duration of exposure the same for the two suspensions. The results were:

		Infectivity titre and, where end-point was not bracketed, infection ratio in eggs inoculated with undiluted sample		
Expt.	Time x (min.)	Dark control	Exposed to 3800 ft.c.	Exposed to 600 ft.c
1	40	3·5	< 0·0, 1/6	< 0·0, 1/6
2	10	1·6	1·3	0·5
	20		< 0·0, 1/7	< 0·0, 3/7

These results are not inconsistent with the hypothesis that the decrease of infectivity of a virus suspension by exposure to light is determined by the product of the intensity and the duration of exposure.

The influence of various factors on susceptibility to inactivation by exposure to light

It has already been shown that filtration is a factor which may influence the susceptibility of a virus suspension to inactivation by exposure to light. The influence of the following factors was also studied.

Incident light intensity. In Expts. 7A–7E, despite the different susceptibilities of suspensions prepared under different conditions, it was observed in all cases that inactivation increased regularly with the intensity of incident light. Inactivation was relatively greater at the lower intensities of illumination.

In Expt. 7D an increase of the incident light intensity from 770 ft.c. to 3800 ft.c. by the insertion of the collimating lens caused the fall of the infectivity titre to increase from 1·0 to 3·0 units. The observation of this greater inactivation, despite the insertion of a lens of 1 cm. average thickness, implied that a residual low intensity of ultraviolet light from the artificial source was not a major factor in the mechanism of inactivation. In Expt. 7E the incident light intensity was modified by the insertion of additional thicknesses of glass as shown in Fig. 1B. With 8 mm. of glass interposed in the collimated beam the incident intensity was reduced from 3800 to 3100 ft.c. The observed decreases of infectivity titre indicated that, if ultraviolet light alone was responsible for inactivation, consideration must be limited to the range of near ultraviolet wavelengths between 3300 and 4000 A. since energy of shorter wavelengths was reduced by at least 95 % by transmission through 8 mm. of the glass.

The addition of certain agents to the suspending medium. There have been many reports of the loss of infectivity of suspensions of viruses following storage for a short period at room temperature. The risk of errors being introduced into infectivity titrations by this instability has been minimized in some laboratories by the use of diluents containing 10 % mammalian serum, or more recently, 0·2 % bovine albumin (Dick & Taylor, 1949). Wilson (1946) used undiluted sheep serum to stabilize the infectivity of suspensions of the virus of louping-ill.

The stabilizing action of 10 % serum on the infectivity of virus suspensions exposed to light was demonstrated strikingly in an experiment in which a suspension of chorioallantoic membrane infected with virus of the Ind. C egg strain of vesicular stomatitis was diluted serially in two equivalent sets using as diluents 0·005 M-phosphate saline with and without the addition of 10 % rabbit serum. Samples of each dilution were exposed simultaneously by the method of multiple dilutions under the conditions of Expt. 4C. The infectivity titres indicated by the control and exposed dilutions were 6·8 and ⩽1·9 in the absence of serum and 7·2 and 6·8 in the presence of serum. Thus the decrease of the indicated titre in the absence of serum was at least 4·0 units greater than in its presence. In Expts. 5A, 5B, 5E and 6F suspensions of the same virus were prepared with 1 and 5 % guinea-pig serum added and only in Expt. 6F was there any suggestion of stabilization of infectivity.

Further demonstration of the value of 10 % serum was obtained in Expt. 7F in which the decrease of titre was at least 4·1 units in the absence of serum but was only 0·3 unit and 1·2 units when 10 % rabbit serum and 10 % guinea-pig serum respectively were present in the suspending medium. Similar protection against inactivation was afforded both when the guinea-pig serum was not inactivated and when it was reconstituted from a freeze-dried sample. The nature of the protective action of serum was investigated further in Expt. 7G where the results suggested that the protective action of serum occurred only when the serum was mixed with the virus suspension and that modification of the incident light by transmission through serum was not the determining factor.

In view of the observation that the infectivity of the virus of equine encephalomyelitis was stabilized by the addition of 1 mg. cysteine hydrochloride/ml. to the suspending medium (Labzoffsky, 1946) the value of this agent was tested in the case of vesicular stomatitis virus. In an experiment made simultaneously with Expt. 3B four sets of multiple dilutions were prepared to which this amount of cysteine hydrochloride was added, followed by readjustment to pH 7·6. The results were in every case the same as those obtained without this reducing agent and indicated no protective action.

Temperature of virus suspensions during exposure. In Expt. 7H there was no significant difference between the decrease of titre observed following exposure at 4° and 20°.

Storage of the initial virus suspension at 4°. A comparison of the results of Expt. 7I with those of Expts. 7A and 7B showed a wide variation in the decrease of infectivity of filtrates of amniotic fluid exposed under identical

Table 7. *Influence of various factors on susceptibility to inactivation. Suspensions exposed in the cell to artificial light*

Expt.	Virus and strain	Infective material from which dilution to be exposed was prepared	Dilution prepared in 0·005 M-phosphate saline for exposure	Temperature and period of exposure	Infectivity titre of control in the dark	Experimental conditions	Incident light intensity (ft.c.)	Decrease of titre due to exposure
(i) Influence of incident light intensity. Samples of single suspensions exposed simultaneously to different intensities of illumination adjusted by the methods detailed.								
A	Vesicular stomatitis Ind. C	Chick embryo amniotic fluid filtered (0·6 μ.) and stored for 10 weeks at 4°	1 in 100	4°, 2 hr.	3·9	Neutral density filters attached to face of cell as shown in Fig. 1 A	3800	3·8
							600	1·6
							38	0·5
B	Vesicular stomatitis Ind. C	Same filtrate as in Expt. A stored for 11 weeks at 4°	1 in 1000	4°, 1 hr.	2·5	As in Expt. A	3800	⩾3·0
							600	1·3
							38	0·7
				4°, 2 hr.	2·8	As in Expt. A	3800	⩾3·3
							600	2·1
							38	⩽0·4
C	Newcastle disease	Unfiltered chick embryo amniotic fluid stored for 6 weeks at 4°	1 in 3200	4°, 4 hr.	2·9	As in Expt. A	3800	⩾3·4
							600	1·3
							38	0·6
D	Vesicular stomatitis Ind. C	1/25 suspension of mouse brain in a mixture of equal parts of 0·005 M-phosphate saline and Hartley's digest broth, freshly filtered (0·6 μ.)	1 in 4	4°, 4 hr.	4·1	Two identical cells and light sources used for concurrent exposure in collimated (1) and uncollimated (2) beams. Cell-source distance 25 cm.	3800 (1)	3·0
							770 (2)	1·0
E	Vesicular stomatitis Ind. C	Fresh filtrate prepared as in Expt. D	1 in 4	4°, 4 hr.	4·1	Various extra thicknesses of glass interposed between cell and light source as shown in Fig. 1 B	3800	2·2
							3100	1·1
							2400	0·7

(ii) Influence of addition of serum to suspending medium. Simultaneous exposure of equivalent dilutions prepared in the media detailed

						Serum added:		
F	Vesicular stomatitis Ind. C	Chick embryo amniotic fluid unfiltered and stored 18 weeks at 4°	1 in 100	20°, 2 hr.	3·6	None	3800	≥4·1
					3·5	10 % rabbit serum heated 56° for 1 hr.	3800	0·3
					3·7	10 % guinea-pig serum heated 56° for 1 hr.	3800	1·2
					3·7	10 % guinea-pig serum unheated	3800	1·1
					3·3	10 % guinea-pig serum reconstituted from freeze-dried serum and heated 56° for 1 hr.	3800	0·7
G	Vesicular stomatitis Ind. C	Fresh filtrate of mouse brain suspension prepared as in Expt. D	1 in 10	4°, 2 hr.	5·8	Suspensions exposed in three cell compartments as shown in Fig. 1C Compartment 2	3100	1·7
						Compartment 1	3100	1·3
					5·4	Compartment 3	3100	0·5

(iii) Influence of temperature of virus suspension during exposure and of storage of initial infective material at 4°

H	Vesicular stomatitis Ind. C	Filtrate of mouse brain suspension used in Expt. D, and stored for 13 days at 4°	1 in 4	4°, 2 hr.	2·9	Suspension exposed at 4°	3800	2·6
			1 in 4	20°, 2 hr.	2·7	Suspension exposed at 20°	3800	1·9
		Filtrate of mouse brain suspension prepared as in Expt. D and stored for 1 day at 4°	1 in 4	20°, 2 hr.	4·9	Suspension exposed at 20°	3800	3·0
I	Vesicular stomatitis NJ. M	Freshly collected chick embryo amniotic fluid	1 in 100	4°, 1 hr.	5·0	Low-speed supernatant	3800	0·2
					3·8	Low-speed supernatant, filtered 0·6 μ.	3800	0·5
					3·2	Low-speed supernatant, filtered Seitz K	3800	1·0
J	Vesicular stomatitis NJ. M and Ind. C	0·6 μ. filtrates of chick embryo amniotic fluid	1 in 100	4°, 1 hr.	3·2	Filtrate of Expt. I, stored for 3 weeks at 4°	3800	≥2·6
					≤3·6	Freshly prepared filtrate, NJ. M strain	3800	≤0·3
					5·8	Freshly prepared filtrate, Ind. C strain	3800	1·4

(iv) Influence of degree of aeration of suspending medium

K	Vesicular stomatitis Ind. C	Chick embryo amniotic fluid unfiltered and stored 19 weeks at 4°. Same source as in Expt. F	1 in 100	20°, 2 hr.	3·8	Dilutions exposed and titrated in unaerated buffer	3800	≥4·3
					3·5	Dilutions exposed and titrated in aerated buffer	3800	≥4·0
		Freshly collected chick embryo amniotic fluid	1 in 100	20°, 2 hr.	5·3	Dilutions exposed and titrated in unaerated buffer	3800	5·3
					5·0	Dilutions exposed and titrated in aerated buffer	3800	≥5·5

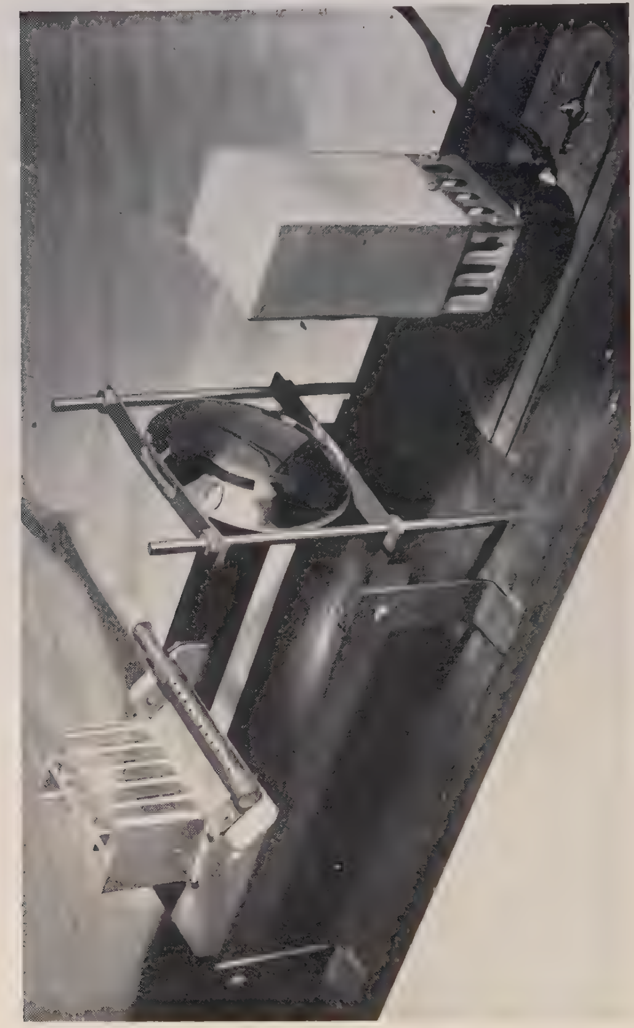
conditions. Two factors possibly contributing to this result were the virus strain used and the period of storage of the filtrate. These factors were investigated in Expt. 7J in which the filtrate of Expt. 7I was used again after a period of storage during which there was no significant change of infectivity. Exposure under the same conditions as before, however, now caused a decrease of titre which was at least 2·1 units greater. The decrease in titre of suspensions prepared from fresh filtrates of either strain and exposed simultaneously again did not exceed 1·4 units. This suggested that dilutions of freshly prepared filtrates of infective amniotic fluid were less susceptible to inactivation by exposure to light than those of filtrates stored for a period at 4°. A comparison of the results of Expts. 7D and 7H with a single filtrate of mouse brain suspension also suggested that susceptibility to inactivation was greater after storage. It was apparent, however, from the results obtained in Expt. 7H at 20° that different fresh filtrates might differ in their susceptibility to inactivation.

Degree of aeration of the suspending medium. An uncontrolled factor which might have contributed to different results in separate experiments was the degree of aeration of the suspending medium. The greater susceptibility of filtered suspensions might also have been due to a decrease in the amount of dissolved gas following filtration by negative pressure. In Expt. 7K a comparison was made between two samples of freshly autoclaved phosphate saline. One of these was saturated with air by shaking and the other was not exposed to the air before use. These two diluents were used to prepare the exposed suspensions and the dilutions for the subsequent titrations. The magnitude of the observed decreases of titre demonstrated the extreme susceptibility of some suspensions even when prepared from fresh material. There was, however, no indication that the degree of aeration of the diluent was a significant factor in the mechanism of inactivation.

DISCUSSION

The aim in this investigation was to determine whether a significant decrease of infectivity occurred when virus suspensions were exposed for relatively short periods to the intensities of visible light normally experienced in the laboratory. Although significant inactivation was demonstrated under these conditions the data obtained indicated that many factors influenced the extent to which suspensions of a given virus might be inactivated. When consideration is limited to a single strain of one virus, known factors which influence the susceptibility to inactivation are the source of infective tissue and the method of preparation and storage of the initial suspension. It is doubtful whether other investigators have considered exposure to light as a factor contributing to the instability of the infectivity of virus suspensions prepared in protein-free media. The authors are unaware of any other investigations in which direct comparisons have been made between the infectivity of virus suspensions placed in light and in darkness.

Although in the present studies it has been shown that serum is of value in maintaining the infectivity of suspensions of vesicular stomatitis virus in the presence of light, there are many instances, particularly in physical-chemical



H. H. Skinner & C. J. Bradish—Inactivation of virus suspensions by light. Plate 1 studies, in which it is required to concentrate and purify specific components and in which the addition of extraneous non-dialysable materials is inadmissible. Other studies at this institute of the virus system of vesicular stomatitis (Bradish, Brooksby & Dillon, unpublished) have shown that it is possible to conduct all manipulations of virus suspensions in rooms illuminated only by photographic safe lights. In this way inactivation by exposure to light is minimized at every stage of the experimental procedure and a quantitative recovery of infectivity may be achieved.

The exact wavelengths to which suspensions of a given virus may be most susceptible have not been investigated. In the experiments with the artificial source of light the effects observed were produced by exposure to light of those wavelengths which are normally present in daylight after passage through glass. The results of the experiments in which the incident light intensity was modified by transmission through additional thicknesses of glass indicated that residual low intensities of ultraviolet light were not a major factor. Further elucidation of the mechanism of the inactivation of virus suspensions by exposure to light requires detailed investigation of the susceptibility of individual virus systems to radiation of defined wavelengths and consideration of the possibility that inactivation may be due to indirect rather than to direct action.

Acknowledgement is due to the Veterinary Laboratory, Ministry of Agriculture and Fisheries, Weybridge, for the viruses of Newcastle disease and of fowl-plague, to the Lister Institute of Preventive Medicine, Elstree, for the virus of vaccinia and to our colleague Dr W. M. Henderson for the strains of influenza virus which he obtained from The Children's Hospital, Philadelphia. Nagel's strain of foot-and-mouth disease virus was received from Prof. Levaditi, Institut Alfred Fournier, Paris.

It is a pleasure to acknowledge the technical assistance of Mr E. H. Knight.

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EXPLANATION OF PLATE

The apparatus employed for the exposure of virus suspensions to the light of the artificial source. Components from right to left: artificial source of light, condenser lens of 10 cm. diameter, cold air blower, exposure cell of five compartments.

(*Received* 15 *October* 1953)

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Antibody Production in Avian Embryos and Young Chicks

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SUMMARY: The inoculation of killed *Salmonella pullorum* into the developing avian embryo on or after the 17th day of incubation or of live *Sal. pullorum* into young chicks evoked no immediate antibody response. Approximately 20–40 days after hatching, however, agglutinins and non-agglutinating antibodies were sometimes detected in the sera of these chicks. Maximum antibody production did not occur until the chicks were approximately 100 days old.

The intravenous inoculation of killed *Sal. pullorum* into developing embryos on or before the 15th day of incubation stimulated the production of practically no demonstrable antibody by the chicks during approximately the first 80 or 100 days after hatching. These chicks, however, showed a marked decrease in capacity to produce demonstrable antibodies in their sera after subsequent oral infection with *Sal. pullorum.*

These experimental results are discussed in relation to various theories of antibody production. It is suggested that they are evidence in support of Burnet's adaptive enzyme theory. The results are explained on the hypothesis that the introduction of antigenic material into the embryo under suitable experimental conditions enables the host cells to become adapted to the antigen, resulting in a diminished antibody response by the host, during post-embryonic life, to a subsequent challenge with similar antigenic material.

A variety of antigenic materials has been employed by earlier workers in unsuccessful attempts to demonstrate the production of antibodies by the developing avian embryo and young chick. These antigens have included diphtheria toxin (Grasset, 1929), *Clostridium sporogenes* (Weinberg & Guelin, 1936), and *Escherichia coli* (Rywosch, 1907). Rywosch claimed that the blood sera from developing embryos of 14–18 days incubation were bactericidal for *E. coli* but to a lesser extent than serum from an adult bird. It was probable that some, at least, of the bactericidal effect of embryonic serum was associated with bactericidins passively transferred from the hen to the egg. Sherman (1919*a*, *b*) found that complement did not appear in embryo serum until the 17th day of incubation, and that lysins for rabbit erythrocytes were not formed until the embryo was about to hatch. Polk, Buddingh & Goodpasture (1938) confirmed Sherman's results on the development of complement, and found that young chicks do not possess normal haemagglutinins and do not respond to immunization with foreign red cells until they have spontaneously developed some agglutinating capacity.

The responses of the embryo to viruses and to tumour material have also been investigated. No antibody was detected in the sera of chicks hatched from eggs infected with influenza virus (Burnet, 1941), bacteriophage

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(Beveridge & Burnet, 1946), or with washed human red cells (Burnet, Stone & Edney, 1950). Moreover, there was no demonstrable change in the subsequent response of the hatched chick to the corresponding antigen. Fox & Laemmert (1947) failed to detect antibodies to yellow fever virus in the embryo, but they observed antibody only in those chicks in which the virus persisted in the blood stream until hatching time. Response of the embryo to implanted tumour tissue did not occur before the 18th to 21st day of incubation (Murphy, 1913, 1914, 1916; Stevenson, 1917, 1918).

Although a wide variety of antigenic materials has been used in experiments to observe the capacity of the embryo and young chick to develop its own antibodies, the sera have been tested by techniques which only detect the presence of complete (divalent or multivalent) antibodies. While it is generally accepted that the mechanism for the production of complete antibodies is absent in the embryo and does not develop until later in life, it is not known whether the development of the antibody-producing mechanism passes through a stage when it can produce only incomplete antibodies in the embryo and newly hatched chick.

In the experiments recorded here, sera from embryos and young chicks have been tested for agglutinins and for non-agglutinating antibodies to *Salmonella pullorum*, to observe whether the non-agglutinating antibody was produced at an earlier stage in development than the agglutinins.

MATERIALS AND METHODS

The methods of preparing suspensions of *Sal. pullorum* and of carrying out tests for agglutinins and non-agglutinating antibodies have already been described (Buxton, 1951, 1952). Suspensions of *Sal. pullorum* for inoculation into developing embryos were washed twice in saline to remove all traces of ethanol and then resuspended to the required concentration. A fresh suspension was prepared for inoculating each batch of eggs.

Various routes of inoculation were employed, particularly the intravenous and yolk-sac methods, using the techniques described by Beveridge & Burnet (1946). A 1·0 ml. all-glass syringe was used with No. 27 needle. The dosage was 0·1 ml. per embryo. In some experiments newly hatched chicks were infected orally and the dose for each chick consisted of 0·1 ml. of an 18 hr. broth culture of *Sal. pullorum* in peptone broth.

The preparation and use of anti-livetin serum for the detection of non-agglutinating antibody in sera was described earlier (Buxton, 1952).

RESULTS

Antibody production in the developing embryo

Initial experiments consisting of the inoculation of minute doses of live *Sal. pullorum* into developing embryos proved fatal on all occasions, and subsequently suspensions of killed bacteria were used.

Intravenous inoculation. Two unsuccessful attempts were made to demonstrate agglutinins and non-agglutinating antibodies in the sera of newly hatched chicks after they had been inoculated intravenously during incubation with killed *Sal. pullorum*. In the first experiment twelve 11-day embryos each received 9×10^7 organisms, and in the second experiment a similar number of 11-day embryos each received 46×10^9 organisms. Immediately after hatching the chicks were bled from the heart and the sera tested in dilutions beginning at 1/5. In no case was any antibody demonstrable.

The observations of Sherman (1919*a*) and of Polk, Buddingh & Goodpasture (1938) that complement could not be detected in embryo serum until at least the 17th day of inoculation, and also that at about that time the embryo suddenly developed the ability to react to the implantation of foreign tissue, suggested that inoculation of bacterial antigen into the embryo on or after the 17th day of incubation might result in an antibody response which is not apparent after inoculation of antigen as early as the 11th day. Fifteen 19-day embryos were inoculated intravenously with approximately 9×10^4 killed bacteria. Eight of these embryos hatched satisfactorily and their sera were tested at intervals during the subsequent 173 days. A group of healthy control eggs from the same source was incubated and hatched with the experimental eggs. Four control chicks were kept with the experimental ones and their sera tested. All chicks were kept strictly isolated throughout the experiment, during which time no antibodies were demonstrated in the sera of the controls. During the period of observation one experimental chick received a mechanical injury and had to be killed. The results are shown in Fig. 1. As in previous trials no antibody was detected in the sera of experimental chicks immediately after hatching. At 20 days of age non-agglutinating antibody was demonstrable in five sera (1/10, 1/20, 1/40, 1/20, 1/10). On the 34th day non-agglutinating antibody (1/80, 0, 1/240, 1/240, 1/40, 0, 1/160, 0) in two of five positive experimental sera showed a well-marked zone, extending in each case from dilutions of 1/40 to 1/160. On the 72nd day the antibody had completely altered in character and all sera contained agglutinins at dilutions of about 1/125 (1/80, 1/160, 1/100, 1/160, 1/160, 1/160, 1/50). Thereafter, there was a marked fall in antibody titres. It was noticeable that at the final test on the 173rd day the non-agglutinating antibody demonstrable in all sera (1/40, 1/20, 1/40, 1/80, 1/40, 1/40, 1/40) did not show any zoning as in earlier tests.

Yolk-sac inoculation. For antibody production to occur in the young chick as a result of inoculation of bacterial antigen *in ovo*, the bacteria may have to maintain their antigenicity until such time as the defence mechanism of the chick has developed sufficiently to respond to the antigenic stimulus. Masurier, Branion & Marcellus (1946) found that in many chicks the yolk-sac was retained for a variable time after hatching. For these reasons a suspension of killed *Sal. pullorum* was inoculated into the yolk-sac of developing embryos to observe whether there was any antibody response by these embryos after hatching. Each of twelve 9-day old embryos was inoculated into the yolk-sac with approximately 23×10^5 killed bacteria. Six chicks hatched successfully and were kept isolated for a period of 123 days, during which time serum samples were tested for agglutinins and non-agglutinating antibodies. Four uninoculated control chicks from the same source of eggs were hatched and kept with the experimental chicks. Their sera remained negative throughout the period of observation. The results of this experiment are shown in Fig. 2. As in the previous experiment, zoning of non-agglutinating antibody occurred before the peak of antibody production, which was delayed until about the 125th day after hatching. (Agglutinating antibody titres: 1/20, 0, 1/30, 1/80, 1/40, 1/5. Non-agglutinating antibody titres: 1/40, 0, 1/40, 1/375, 1/375, 1/50.)

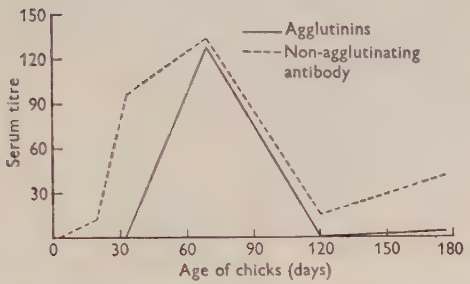

Fig. 1. Antibody production by young chicks which had been inoculated intravenously with killed *Sal. pullorum* on the 19th day of incubation.

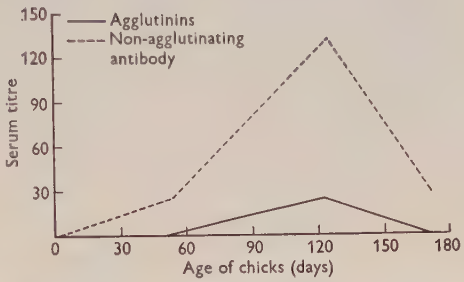

Fig. 2. Antibody production by young chicks which had been inoculated into the yolk-sac with killed *Sal. pullorum* on the 9th day of incubation.

Antibody production in the young chick

Intraperitoneal infection. Ten 4-day healthy chicks were inoculated intraperitoneally with an 18 hr. broth culture of *Sal. pullorum.* Each chick received a dose of approximately 2×10^7 organisms in 0·1 ml. of diluted culture. Three chicks died within a few days of inoculation and the sera from the remainder were tested at intervals for agglutinins and non-agglutinating antibodies. A 4th chick died from a generalized infection of *Sal. pullorum* soon after the 102nd day of the experiment. The results presented in Fig. 3 show that, as in the case of chicks which had received killed bacteria *in ovo*, non-agglutinating antibody appeared first and rose to a higher titre than agglutinins. (Agglutinating antibody: 1/80, 1/80, 1/60, 1/1920, 1/40, 0, 1/1280. Non-agglutinating antibody: 1/100, 1/320, 1/160, 1/3840, 1/160, 0, 1/8320.) Two chicks showed marked zoning of agglutinins on the 55th and 102nd days of the experiment. A similar experiment was repeated on four more chicks which survived intraperitoneal infection and the results are similar to those in Fig. 3, namely that non-agglutinating antibody appeared first and increased to a higher titre than the agglutinins.

Oral infection. Each of ten chicks received a dose of approximately 2×10^7 live *Sal. pullorum* at one day of age, and their subsequent antibody production is shown in Fig. 4. In general, antibodies consisted mainly of the non-agglutinating type and maximum titres were lower than those resulting from intraperitoneal inoculation of live bacteria. Nevertheless, the pattern of antibody response was similar to the previous experiment in two respects, namely, that non-agglutinating antibody was detected earlier than agglutinins and that it reached higher titres than the latter. (Agglutinating antibody: 1/20, 1/5, 0, 1/5, 0, 1/10, 0, 0, 1/20, 0. Non-agglutinating antibody: 1/40, 1/40, 1/20, 1/10, 1/100, 1/50, 1/10, 1/20, 1/40, 1/80.)

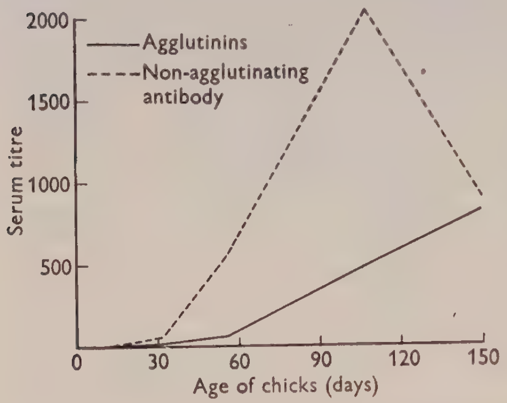

Fig. 3. Antibody production by young chicks which had been inoculated intraperitoneally with live *Sal. pullorum* on the 4th day after hatching.

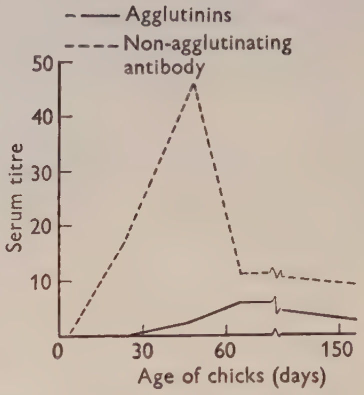

Fig. 4. Antibody production by young chicks which had been infected orally with *Sal. pullorum* on the 1st day after hatching.

The effect of inoculation of antigen in ovo *on the subsequent development of antibodies in the chick*

Inoculation of living antigen intraperitoneally or orally into newly hatched chicks resulted in antibody production which was only demonstrable after a period varying from 11 to 30 days following inoculation. Similarly, the inoculation of killed antigen into the developing embryo under suitable experimental conditions also resulted in antibody production in the chicks which hatched from these eggs. After intravenous inoculation into the embryo on the 19th day of incubation, agglutinins were not uniformly produced by all the hatched chicks until they were at least 20–30 days old. During this lag phase between the time the embryos were inoculated and the time of appearance of antibodies in the hatched chicks' sera, the necessary conditions for the production of detectable antibodies must have been developed. In their theory on the development of marker recognition in embryonic life and its relationship to antibody production, Burnet & Fenner (1949) were of opinion that following a generalized non-fatal infection of the embryo *in utero* by a pathogenic micro-organism, the animal after birth would be incapable of responding by antibody production to injection or infection with the same type of micro-organism. Thus, if developing embryos were inoculated intravenously with killed *Sal. pullorum* and after hatching the chicks were again subjected to contact with this antigen, the subsequent antibody production would be less than in chicks which had not previously been inoculated *in ovo*. The testing of this possibility was the object of the following experiments.

First experiment. A total of forty-five embryonated eggs laid by known healthy Brown Leghorns was divided into three groups of 15 eggs each. After

9 days of incubation one group of eggs was inoculated in the yolk-sac with approximately 25×10^4 killed *Sal. pullorum* organisms suspended in 0·1 ml. saline. After 15 days of incubation the second group of eggs was inoculated intravenously with a similar dose. The third group of 15 embryonated eggs was kept as uninoculated controls. After hatching, there was available a total of 10 chicks in the yolk-sac inoculated group, 8 chicks in the intravenously inoculated group, and 7 chicks in the control group. The groups were kept strictly isolated in a separate chick-rearing room and all groups were maintained under identical conditions of management. When the chicks were 27 days old their sera were tested for agglutinins and non-agglutinating antibodies. All were negative at 1/5 and upwards.

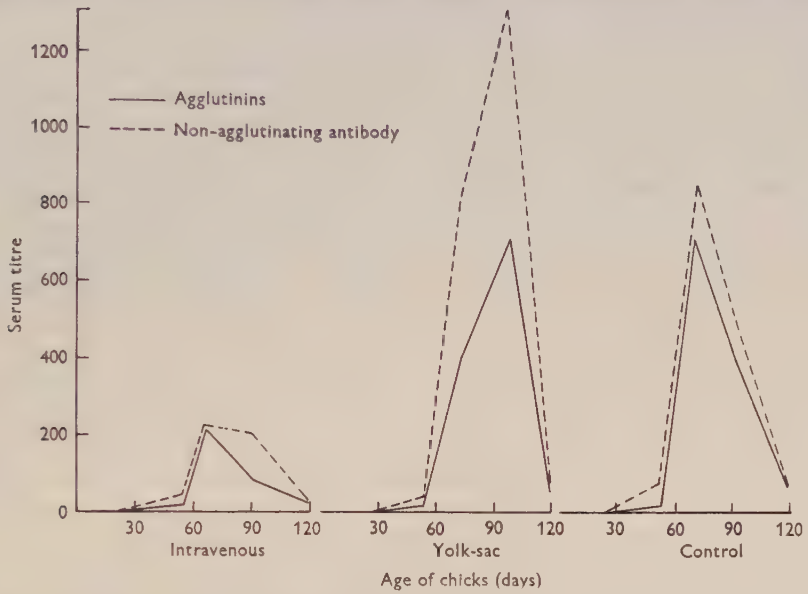

Fig. 5. Average antibody production in groups of chicks which had been inoculated intravenously or into the yolk-sac *in ovo* with killed *Sal. pullorum*, and later infected orally with *Sal. pullorum* 34 days after hatching.

When 34 days old, each chick was infected orally with 0·1 ml. of an 18 hr. broth culture of *Sal. pullorum*. Subsequently the chicks were tested for antibodies at 54, 68, 90 and 118 days old. These tests showed (Fig. 5) the average serum titre for each group of chicks. By the 68th day (i.e. 34 days after infection) the average titres of agglutinins (1/80, 1/150, 1/2250, 1/20, 1/2000, 1/500, 1/20) and non-agglutinating antibodies (1/160, 1/300, 1/2500, 1/80, 1/2250, 1/640, 1/80) in the control group had risen to approximately 1/700 and 1/850 respectively, while the average serum titre for non-agglutinating antibodies (1/80, 1/1280, 1/80, 1/10, 1/160, 1/10, 1/10, 1/40) was only about 1/200 in the group which had previously been inoculated intravenously with antigen on the 15th day of incubation. There was little difference between the control group and the group previously inoculated with antigen into the yolks-sac on the 9th day of incubation, except that the non-agglutinating antibody titre of the control group was lower than that in the latter group. The results showed that under these experimental conditions the inoculation of bacterial antigen intravenously into developing embryos on the 15th day of incubation decreased the capacity of the hatched chicks to produce demonstrable antibodies after subsequent infection with live organisms at 34 days of age.

Second experiment. The apparent diminution of antibody response in hatched chicks to infection after preliminary intravenous inoculation *in ovo* with killed homologous antigen, might depend largely on the developmental stage of the antibody-producing cells in the embryo at the time of inoculation. The only immune responses ever observed in the avian embryo have been to the implantation of foreign tissue during the 18th to 21st days of incubation. This, together with the appearance of complement on or after the 17th day, suggested on the basis of Burnet's hypothesis that during these last 4 or 5 days of incubation the balance of intracellular enzyme systems in the embryo becomes less labile and, therefore, more able to respond to the implantation of foreign tissue. At an earlier stage in development, however, the embryo may be able to adapt itself to the introduction of bacterial antigen and the demonstrable response of the hatched chick to subsequent contact with the same antigen might thereby be decreased. The following experiment was carried out in an attempt to confirm this view.

A group of 19 chicks was used; 6 had been inoculated with killed *Sal. pullorum* on the 15th day of incubation, 6 on the 20th day, and the remaining 7 had been inoculated on both the 15th and 20th days of incubation. The inoculum was given intravenously and the dosage in each case was approximately 25×10^4 killed bacteria suspended in 0·1 ml. saline. The chicks were kept strictly isolated. On the 45th day after hatching, each chick was infected orally with 0·1 ml. of an 18 hr. broth culture of live *Sal. pullorum.* Subsequently the sera were tested for agglutinins and non-agglutinating antibodies. The average serum titres for each group of chicks are shown in Table 1. There

Table 1. *The average antibody production of groups of chicks inoculated intravenously with killed* Salmonella pullorum *during incubation and subsequently infected orally with live* Sal. pullorum *when 54 days old*

The figures in the table are the reciprocals of the antibody titre. A = agglutinins; NA = non-agglutinating antibody.

	Age of embryos when inoculated (days of incubation)					
Age of chicks (days)	15		15+20		20	
	A	NA	A	NA	A	NA
61	21	45	6	48	32	88
67	32	213	16	50	462	3082
74	25	30	18	40	3840	6400
82	15	20	18	180	5120	25600
88	30	60	20	70	960	3840
95	15	20	18	35	1600	3840
102	nt	nt	17	60	1280	5120
109	20	30	20	80	640	1280

nt = not tested.

is a striking contrast between the titres of those chicks which were originally inoculated on the 20th day of incubation, and those inoculated on the 15th or 15th and 20th days. The results of this and the previous experiment indicate that in order to demonstrate Burnet's hypothesis of embryonic adaptation to antigenic material, it is necessary to inoculate bacterial antigen intravenously into the embryo before the 20th day of incubation. The 15th day of incubation was found satisfactory.

Third experiment. It was considered possible that under these conditions the degree of adaptation might depend on the size of the initial dose of antigen inoculated *in ovo*, and that a dose in excess of the optimum might result in some of the inoculum remaining antigenically unaltered until the embryo had reached the stage in development when it could respond to the antigenic stimulus by the production of antibodies. This might be observed by either relatively high antibody titres in the hatched chicks when compared with uninoculated controls, or titres might be higher in those chicks after they had been subsequently challenged with a further dose of antigen than in ones which received only the initial dose *in ovo*.

Preliminary experiments showed that the inoculation of a sufficiently large dose of killed bacteria was toxic to the embryo. For this reason four groups of embryos were inoculated and each received a dose of 25×10^7, 25×10^6, 25×10^5, or 25×10^4 killed bacteria intravenously on the 12th day of incubation. The total number of chicks hatched from these four groups were 14, 10, 12 and 12 chicks, respectively. When the chicks were 35 days old their sera were tested for agglutinins, and half the number of chicks in each group was infected orally with 0·1 ml. of an 18 hr. broth culture of *Sal. pullorum*. The uninfected chicks were isolated. A total of 16 control chicks, uninoculated *in ovo*, were also divided into two groups, one group being infected when 35 days old. The sera from all chicks were tested for agglutinins during the subsequent 80 days. The results are shown in Fig. 6. Uninfected control chicks remained negative. There was no significant difference between the average titres of the groups of experimental chicks infected when 35 days old, and these titres were markedly lower than the corresponding average titres of the control infected chicks. The group receiving *in ovo* inoculation of 25×10^5 organisms responded with an average maximum titre of 1/10 on the 36th day after infection. These results show that the intravenous inoculation of killed *Sal. pullorum* into the developing embryo on the 12th day of incubation did not result in subsequent agglutinin production by the hatched chicks, and that such chicks when later challenged orally with live *Sal. pullorum* produced markedly lower agglutinin titres than control infected chicks. Moreover, the range of doses of killed organisms inoculated *in ovo*, resulted in no significant difference between the antibody response of the different groups of chicks.

DISCUSSION

The experimental results have shown that the inoculation of killed *Sal. pullorum* into the avian embryo on the 11th day of incubation failed to stimulate the production of demonstrable antibodies in the newly hatched chicks;

and that similar inoculation of antigen on the 12th day of incubation did not result in agglutinin production by the chicks at least during the first 100 days after hatching. After the oral infection of 4-day chicks with *Sal. pullorum*, antibodies were not detected until the chicks were 20–40 days old; maximum antibody production did not occur after intraperitoneal inoculation until the chicks were approximately 100 days old. These latter results correspond to those of Norton, Wolfe & Crowe (1950) who used crystalline beef albumin or undiluted beef serum as antigen, and who found that there was some correlation between the size of spleens in the young chicks and their capacity to produce demonstrable antibodies.

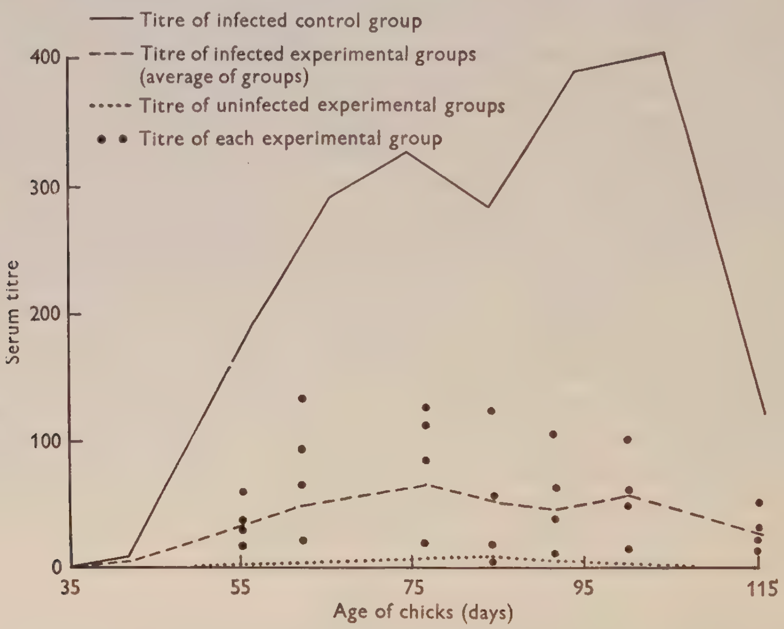

Fig. 6. Average agglutinin production of groups of chicks which had been inoculated intravenously with different doses of killed *Sal. pullorum* on the 12th day of incubation, and subsequently infected orally with *Sal. pullorum* on the 35th day after hatching.

The first appearance of detectable agglutinins at relatively long intervals after administration of antigenic material is an indication of the time taken for the development of the antibody producing mechanism. There is some evidence, however, that the host reacts to the presence of antigen before the production of agglutinins. This reaction may take the form of the production of non-agglutinating antibody alone, or diminution of the hosts' ability to produce agglutinins after subsequent contact with the homologous antigen. The absence of agglutinins in the serum of young chicks which have previously been inoculated with bacterial antigen does not necessarily imply a total lack of antibody response by the host. On the contrary, the presence or absence of agglutinins may be only an indication of the state of development of the host's cells responsible for the production of antibodies. Burnet & Fenner

(1948) believed that the type of antibody produced might differ according to the type and stage of specialization of the cells responsible for its production. Brandt, Clegg & Andrews (1951) examined in detail the quantitative and qualitative change of serum globulin fractions during the young chick's development to maturity, and the results give some indication of the rate at which maturation of the antibody-producing cells proceeds. They found that the values for globulin varied according to the age of the bird and that they increased from 0·74 g./100 ml. in 4- to 7-week-old chicks to 1·3 g./100 ml. in laying hens. The values for albumin and β-globulin showed little variation. Thus, in the young chick a proportion of the globulin-producing cells is immature. In such cells which are undergoing development, the pattern of the balance existing between the intracellular enzymes may be in various stages of development towards the final form. According to Burnet & Fenner's theory, the lability of intracellular enzymes is lost as the young host matures, and is later replaced by a permanent balance which remains throughout adult life. Until this final balance is reached it seems improbable that contact between the cell and foreign antigen will stimulate the production of complete antibody molecules (i.e. agglutinins) in which each molecule possesses two or more combining sites.

Although the intravenous inoculation of bacterial antigen into developing embryos on or before the 15th day of incubation did not stimulate the production of antibodies in the hatched chick, the capacity of these chicks later in life to produce demonstrable antibodies, when challenged again with similar bacterial antigen, was diminished. In the opinion of Burnet & Fenner (1949) '...the primary units (adaptive enzymes) on which antibody production is based, are modifications of enzyme systems primarily adapted to specific adaptation to one or other of the self-marker components of the body cells. When and only when, the specific adaptations occur, the marker is enzymatically destroyed without disturbance of intracellular equilibrium'. These primary units (adaptive enzymes) may be the only form of response which the embryo or newborn is capable of producing. Although the presence of primary units in sera would not be demonstrable by the customary *in vitro* techniques for the demonstration of antibodies, they may be responsible for the decreased capacity of the host to produce demonstrable antibodies to the particular antigen later in life, provided that the pattern of the enzymes can be adapted to fit the configuration of the substrate (i.e. the antigenic components of *Sal. pullorum*).

Burnet *et al.* (1950) were unable to demonstrate antibodies in chick embryos after inoculation *in ovo* of either influenza virus or bacteriophage. Moreover, the inoculation of these antigens did not alter the response of the hatched chicks to subsequent immunization with similar antigens. These results are at variance with those reported here using a bacterial antigen. Burnet and colleagues did not inoculate their experimental materials intravenously into the developing embryo; with *Sal. pullorum* the intravenous route was the only one which yielded any response by way of a diminished ability of the young chick to produce antibodies later in life. On the basis of the theory already stated, it is reasonable to expect that early contact between developing cells of the embryo's reticulo-endothelial system and unaltered antigen are essential conditions for the demonstration of this phenomenon, and that these conditions would be maintained provided that the inoculations were made intravenously. The detection of antibody in Burnet's experiments required the production by the embryo of virus antihaemagglutinins and phage-inactivating antibodies. Each of these may be considered as complete types of antibody in that they appear as the result of an adult host's response to contact with the respective antigens and are the products of the stimulation of the mature reticulo-endothelial system. Under the experimental conditions described, if the embryos had responded by producing the equivalent of non-agglutinating bacterial antibodies or even primary units of antibody (adapted enzymes) these would not have been observed by the *in vitro* tests employed. A further difference between the experimental procedure of Burnet and those reported here is that in the former embryos were inoculated with living particles (virus, bacteriophage) and in the latter they received killed *Sal. pullorum*. Basset (1951) and others emphasized that the intracellular existence of virus and bacteriophage may differ fundamentally in the process of antibody production from that which occurs with bacteria. Particles containing enzymes may be more resistant to enzyme attack than dead particles (Sevag, 1951). In embryos in which enzyme systems are only partially developed the host may be unable to respond as effectively to a stimulus from living antigen as it can from dead antigen. In addition, the enzyme systems of living organisms such as viruses, adapt themselves to their environment (Monod, 1949; Hinshelwood, 1946) so that the cellular enzymes of the young host, which are still in the developmental stage, must respond not only to the stimulus of the original inoculation of living particles, but also to possible adaptation of the particles themselves to their environment in the host. In contrast to the living antigenic materials used by Burnet, the intravenous inoculation of killed bacteria into the embryo resulted in a relatively sudden contact between the host's cells and antigen which is less resistant to attack by cellular enzymes. In addition, dead particles of antigen will be altered only as a result of responsive action by the host's cells which will not be modified by any enzyme activity of the antigen itself.

The experimental results have been discussed in relation to Burnet & Fenner's adaptive enzyme theory of antibody production. Pauling (1940) and Haurowitz (1949), however, are of opinion that antibody production can proceed only so long as antigen remains intact within the host's cells. Confirmation of this hypothesis can only follow the use of techniques which detect the presence of minute quantities of antigen *in vivo*. Limited evidence in favour of this theory has been produced by Herdegen, Halbert & Mudd (1947), who worked with *Shigella paradysenteriae*, and by McMaster & Kruse (1951), who used labelled globulin as antigen. Moreover, it is considered by some that long-lasting immunity associated with virus diseases (e.g. smallpox, yellow fever, measles) may be due to the multiplication in the host of variants to these viruses which do not produce pathological symptoms. The evidence from experiments with *Sal. pullorum,* however, appears to conform more nearly to the adaptive enzyme theory of Burnet, since the inoculation of antigen into the embryo on or before the 15th day of incubation not only failed to stimulate the production of antibodies in the hatched chick, which according to Pauling and Haurowitz suggests that the antigen had been destroyed, but it resulted in a subsequent diminished ability of the chick to produce antibody, possibly because of the adaptation of the host's intracellular enzymes to the antigen.

I wish to express my appreciation of the encouragement I received from Dr R. F. Gordon, Director of the Animal Health Trust's Poultry Research Station, Houghton, Huntingdonshire, where much of this work was carried out. I am also most grateful to Prof. R. E. Glover for his interest and advice in the work which was continued at Liverpool.

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(*Received* 20 *October* 1953)

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On Amylolytic strains of *Streptococcus pyogenes*

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SUMMARY: Strains of *Streptococcus pyogenes* belonging to Types 2 and 4 elaborated an amylolytic enzyme which was not usually a product of other serological Types though it was occasionally produced by members of Types 1, 9, 22 and Types related to 4, 5 and 25. Sixty-six % of amylolytic strains were untypable by M precipitin tests.

The enzyme was produced by variants. Amylolytic organisms were generated by matt and large opaque colonies of the parent strains; small and glossy variants generated relatively fewer enzyme-positive cocci, and had often lost the amylolytic property. When studied as a single character in serial subcultures on starch agar, the property was transmissible, in some instances to 80 % of the progeny for many generations (forty or more subcultures). Other strains readily lost the property in subculture on starch agar but recovered amylolytic power during passage on plasma agar in the absence of substrate. Certain amylolytic strains contained a trypsin-resistant, serologically active substance which precipitated in anti-M, anti-T and anti-proteinase precursor serum. A similar substance diffused into the medium when strains were grown on agar containing the substrate. While serologically related to the cell antigen, the free substance was trypsin-sensitive.

Certain strains of Group A streptococci liberate an enzyme resembling α-amylase (Crowley, 1950). The enzyme is usually found in culture-filtrates of Type 4 strains, which also produce hyaluronidase. The object of the present work was to determine the frequency of amylolytic strains among representative Types of Group A streptococci other than Type 4, already shown to produce α-amylase (Crowley, 1950), and to study factors contributing to the spontaneously acquired amylolytic activity of single strains which did not belong to Type 4. This paper reports the frequency of amylolytic strains among 200 Group A streptococci in relation to serological Type (T and M). It also gives an account of observations made on amylolytic strains in relation to (1) amylolytic properties as a single character of variants, and (2) cellular and 'free' antigenic components of amylolytic cultures.

MATERIALS AND METHODS

Strains. Thirty stock cultures were received either from the Streptococcal Reference Laboratory, Colindale Avenue, N.W. 9 or from Dr R. C. Lancefield, Rockefeller Hospital, New York. Certain stock strains are named in the text. One hundred and seventy strains, 140 from England and 30 from the United States, were recently isolated when received in this laboratory. The majority of British strains were selected by the Streptococcal Reference Laboratory as single representatives of the one 'strain', namely a member of a serological Type causing infection in a particular region. In many instances several other representatives of one strain were also tested, but these are regarded as the same strain and are not included in the total number of 200. Twenty-eight strains from a single source, mentioned on p. 417, were received after the first part of the work was completed; these are not included in Tables 1–3.

Serological tests. Precipitin tests were carried out in capillary pipettes. Cell extracts for routine M typing were made by the method of Swift, Wilson & Lancefield (1943). T antigen extracts were prepared by the method of Pakula (1951). Precipitating and agglutinating anti-sera were supplied by the Streptococcal Reference Laboratory: anti-M sera Types, 1, 2, 3, 4, 5, 6, 9, 12, 14, 15, 17, 18, 19, 23, 24, 26, 28, 29, 30. Anti-T agglutinating sera Types 1, 3, 13, B3264, 2, 4, 24, 26, 28, 29, 46, 6, 5, 11, 12, 27, 44, 14, 8, 25, Imp 19, 15, 17, 23, 30, 47, 19, 9, 18. The anti-sera were from batches usually issued for routine typing of streptococci by precipitation and agglutination, and were not specially absorbed. Both anti-M and anti-T sera may have contained either M, T, R or non-specific antibodies. Anti-M sera for Types 5, 12 and 14, and anti-T precipitating sera for Types 5 and 12 were specially absorbed with heterologous Type extracts. Proteinase-precursor anti-serum was supplied by Dr S. D. Elliott, Rockefeller Hospital, New York. All amylolytic strains, certain amylase negative strains and stock cultures were typed either by precipitin tests, or by slide agglutination (Griffith, 1934). Typing results for other amylase-negative strains were supplied by the Streptococcal Reference Laboratory. Cell extracts for serological grouping were prepared with lytic filtrates of *Streptomyces* sp. (Maxted, 1948).

Culture media. Agar base was prepared with Hartley digest broth, and the same base was used in blood agar, plasma agar and starch agar.

Plasma agar. Plasma agar contained pooled human plasma (20 %, v/v, discarded from blood banks) and Fildes's peptic digest of sheep cells (2·5 %, v/v).

Starch agar. Starch agar ('minimal') contained soluble starch (1 % w/v, from freshly made gel) and Fildes's peptic digest (2·5 %, v/v). Amioca (glutinous corn starch), which was also used in starch agar, was given by Messrs Brown and Polson, Wellington House, Strand, W. 2, or by the American Amaizo Company.

Broth cultures. Broth cultures for M and T typing were made in Neopeptone infusion broth (modified from Todd & Hewitt, 1932) and in buffered peptone medium (Benger's peptone, 2 % w/v, in sterile tap water containing NaCl, 0·5 % w/v, and sodium β-glycerophosphate, 2·5 % v/v). To obtain extra heavy growth of streptococci glucose (0·1 %, w/v) and Fildes's digest (2·5 %, v/v) were also added. The pH value was adjusted to 7·8. Frozen and dried cultures were kept in brain-heart infusion (Difco).

Detection of amylase activity. Broth cultures or cell suspensions were seeded on starch agar to form growth in plaques. The inoculum was transferred with sterile capillary tubing or '50-drop pipettes', which delivered a standard volume to form plaques about 1 cm. in diameter. Six to nine cultures were seeded on one culture plate with an amylase-positive control strain for comparison. After overnight incubation at 37°, the homogeneity, opacity and amount of growth were recorded. Culture plates were then exposed to iodine vapour, and amylase-diffusion zones were indicated by achroic or dextrinized areas around the growth plaques. Doubtful and negative strains were retested by incubating culture plates for 2 or more days. Amylase-positive strains were streaked out on starch agar for several subcultures, and broth culture supernatants containing no substrate were titrated for amylase by iodometric assay (Crowley, 1950).

Proteolytic activity. Proteolytic activity was tested by the method of Elliott & Dole (1947). In order to demonstrate the presence of the inactive proteinase precursor, culture supernatants were titrated for proteinase before and after incubation with (1) sodium mercaptoacetate (0·1 M) at pH 7·0 for 1 hr., 37°, and (2) crystalline trypsin (0·001 mg./ml.) at pH 8·0 for 1 hr., 37° (Elliott, 1950).

Trypsin sensitivity. Samples of antigenic material were incubated with crystalline trypsin (0·01 mg./ml.) at pH 7·8 for 6 hr. at 37°, and after sampling, the digests were re-incubated overnight.

Hyaluronidase production. Hyaluronidase production was assayed by the mucin clot-prevention test (McClean, 1942).

EXPERIMENTAL

Two hundred strains of Group A streptococci were grown on starch agar plates without enrichment other than Fildes's digest. Though not all strains grew well on the medium, it supported the growth of the majority. Certain strains which grew either poorly or indifferently were grown on starch agar prepared with Benger's peptone (2%). All the strains were also grown on blood agar and plasma agar and the degree of haemolysis and the appearance of the colonies recorded. Amylolytic strains formed opaque growth on the 'minimal' starch agar, while the growth of amylase-negative strains was translucent. About half of the amylolytic strains formed homogeneous opaque cultures, but the remaining strains formed cultures in which large opaque colonies predominated, but small translucent colonies were visible through a hand-lens. A few strains gave rise to occasional large opaque amylolytic colonies which stood out against a background growth of translucent microcolonies. After exposure to iodine vapour amylolytic strains were easily classified as strong-positive, weak-positive or doubtful.

Strong-positive strains formed homogeneous opaque cultures with achroic zones of twice the diameter of the plaques, and in subcultures streaked out on starch agar, more than 80% of the colonies were enzyme-positive. The strains also liberated amylase in broth cultures in the absence of substrate, the titres ranging from 1/32 to 1/128 by iodometric assay (Crowley, 1950). *Weak-positive strains* formed heterogeneous cultures, containing strong-positive, weak-positive and negative colonies. Some, but not all, weak-positive strains liberated amylase in broth cultures to a titre of 1/16, but the majority produced little or no enzyme in the absence of starch. Provided broth cultures were neutralized during growth, the addition of substrate (1% v/v) was followed by increased yield of amylase from both strong-positive and weak-positive strains. In these cultural conditions weak-positive strains whilst producing more amylase, were always weak in comparison with strong-positive strains. *Doubtful strains* were those which gave rise to a few enzyme-positive colonies but which did not produce enzyme in subcultures on starch agar, and liberated no enzyme in starch broth. The total number of amylolytic strains among the 200 strains tested was not altered by growing amylase-negative strains in starch broth; starch agar was the more sensitive medium for the detection of weak activity.

Excluding all 'doubtful' results 73 Group A strains produced amylase. The frequency of amylolytic strains is shown in Table 1, distinguishing strains typed by the M precipitin method from those that could not be so typed and were identified by slide agglutination. Table 2 shows members of closely related specific Types (Stewart, Swift, Wilson & Lancefield, 1944; Lancefield & Dole, 1946; Pakula, 1951). Table 3 shows strains belonging to specific Types having single T antigens.

Table 1. *The frequency of amylolytic strains among 200 Group A streptococci*

Amylase + = strains producing enzyme in starch agar cultures. Amylase − = strains producing no enzyme in presence of substrate. % = % of total in each category.

		Amylase +		Amylase −	
Strains	Nos.	Nos.	%	Nos.	%
Group A, total	200	73	36·5	127	63·5
Group A, typed by M precipitin tests	121	25	20·7	96	79·3
Group A, typed by slide agglutination	70	43	62·5	27	38·5
Group A, untypable	9	5	55	4	45

The results show that all members of Type 2, and most members of the Group A Types related to 4, elaborated amylase. Less than 20% of the other Group A strains produced the enzyme, the exceptional strains being found in several different T antigen groups, and also among strains having single T antigens. A survey of amylolytic strains among a truly representative collection was not attempted, because these results established that occasional amylolytic strains were likely to be found among members of any specific Type. On the other hand, with regard to Types 2 and 4, there was a clear indication of relationship between serological Type and amylase-activity.

Anomalies connected with serological typing of amylolytic streptococci

Sixty-six per cent of amylolytic strains were untypable by M precipitin tests. The majority of these, on the basis of their hyaluronidase production (Crowley, 1944) and their agglutination reaction, were assigned to Type 4. Though the reaction was weak seven Type 4 strains were typable by the M precipitin reaction. In this connexion it has been pointed out by Dr R. C. Lancefield and by Mr W. R. Maxted (personal communications) that satis-

Table 2. *The incidence of amylolytic strains among closely related specific Types*

Non-precipitating strains were assigned to different T antigen groups on results of slide-agglutination tests. Type 4 strains produced hyaluronidase. * = Single strains showed weak amylase activity as isolated phenomenon. M± = doubtful precipitation.

T antigen group of closely related specific Types	No. of strains in group	Specific Types of strains with reactive M protein	Nos.	No. of strains	
				Amylase +	Amylase −
3, 13. B3264	16	Type 3	7	0	7
		Type 13	6	0	6
		Non-precipitating	3	0	3
4, 24, 26, 28, 29, 46	40	Type 4	7 (M±)	7	0
		Type 24	3	1	2
		Type 26	1	0	1
		Type 28	2 (M±)	2	0
		Type 29	1	0	1
		Type 46	1	0	1
		Non-precipitating	25	23	2
5, 11, 12, 27, 44	34	Type 5	14	0	14
		Type 12	9	0	9
		Type 44	2	0	2
		Non-precipitating	9	2	7*
8, 25, Imp 19	6	Type 8	1	0	1
		Type 25	1	0	1
		Non-precipitating	4	2	2
14, R491	11	Type 14	10	0	10*
		Non-precipitating	1	0	1
15, 17, 19, 23, 30, 47	18	Type 15	1	0	1
		Type 17	2	0	2
		Type 19	5	0	5
		Type 23	5	0	5
		Type 30	1	0	1
		Non-precipitating	4	0	4

Table 3. *The incidence of amylolytic strains among serological Types having single T antigens*

M = typed by precipitin tests. * = Single strains showed weak activity as isolated phenomenon. All type 22 strains produced hyaluronidase.

Group A serological type	No. of strains	Numbers typable by M precipitin tests		No. of strains	
				Amylase +	Amylase −
2	22	M	13	13	.
		Non-precipitating	9	9	.
22	18	M	4	.	4*
		Non-precipitating	14	4	10
9	7	M	3	.	3
		Non-precipitating	4	3	1
6	7	M	6	.	6*
		Non-precipitating	1	.	1
18	2	M	2	.	2
31	2	M	2	.	2
1	8	M	8	2	6

factory anti-M sera for certain Types including Types 2, 4, 9, 22, 25, 28 are difficult to prepare. Since amylolytic strains largely belonged to these Types, it seemed possible that the amylolytic property was in some way connected with the difficulty of M precipitin typing.

Multiple agglutination reactions

In the course of typing the streptococci by slide agglutination a number of anomalous reactions were observed which suggested the possibility that amylolytic strains elaborated a common antigenic substance which reacted with an antibody present in immune rabbit sera prepared with amylolytic or potentially amylolytic strains. Thus two amylolytic strains identified as Type 1 by precipitation, were agglutinated by a serum for Type 2. Similar cross-reactions were observed with amylolytic strains of Types 4 and 22, 4 and 25, 22 and 14 and 22 and 9.

Loss of M antigen by amylolytic streptococci

The physical, chemical and immunological properties of the M substance have been described by Lancefield and her co-workers (Lancefield, 1940; Lancefield & Dole, 1946; Lancefield & Perlman, 1952). The M substance is the labile Type-specific cell antigen of *Streptococcus pyogenes* which is the major factor contributing to the potential virulence of a strain, and which stimulates protective immunity in the mouse. When extracted from streptococci by boiling the cells at pH 2, the M substance is an alcohol-soluble protein, and its serological activity is destroyed by proteolytic enzymes, notably by trypsin and by a proteinase (Elliott, 1945) produced by Group A streptococci in particular cultural conditions. Two other Type-specific cell components have been found in certain Group A strains which may be mistaken for M antigen, namely the R antigen of Type 28 strains (Lancefield & Perlman, 1952) and the T substance of Type 2 (Maxted, 1953). Both are heat-stable trypsin-resistant antigens, but either property may be modified by the method of extraction. Certain Types 2 and 28 strains may yield T or R antigens in acid extracts and small amounts may form heavy flocculent precipitates in homologous Type anti-M serum if this is not specially absorbed. It may be noted here that amylase-positive Types 2 and 28 strains, which were typable by the routine method, contained a cell antigen which was readily extracted by trypsin, though not by pepsin, and also by heating at 100°, pH 7·8. These extracts formed very heavy flocculent precipitates in homologous Type anti-M serum which may have been mistaken for M antigen. In view of these findings it was necessary to distinguish between strains typable by M precipitin tests and strains having M protein with the properties described by Lancefield. On the other hand, the results obtained for a limited number of amylase-negative strains (Types 1, 5 and 12) which were typed by the routine method, suggest that these contained M protein which was acid-soluble, heat-stable, trypsin-sensitive and not extractable by any other method.

Amylase production by strains of related specific Types 5, 11, 12, 27, 44

The results in Table 2 show that of the strains representing closely related specific Types 5, 11, 12, 27 and 44, two were amylase-positive and both were untypable by M precipitin tests. In the same connexion tests for amylase were made on twenty-eight strains isolated in one district which were received from the Streptococcal Reference Laboratory. Because these may have been of one strain, and it has not yet been shown that they were not, the strains were omitted from the total 200 representative Group A Types. Table 4 shows that eleven amylase-positive strains were untypable by M precipitin tests and liberated larger amounts of the inactive proteinase precursor than any of the other strains; the latter produced little or none in Neopeptone broth cultures used for M typing. Like the Type 5 strains shown in Table 2, the seven strains identified as Type 5 by M precipitin tests were amylase-negative. However, a further seven strains, also without M protein were amylase-negative and of these two were indistinguishable by colony character from the eleven amylolytic strains shown in Table 4.

Table 4. *Amylase production by certain strains in the T antigen group of closely related Types* 5, 11, 12, 27, 44

The results of T precipitin tests (Types 5 and 12) were inconclusive. Inactive proteinase precursor + +, ±, indicates strain differences in proteolytic activity of filtrates after conversion (Neopeptone broth). Ten amylase-positive cultures were homogeneous, and the eleventh contained less than 40 % of amylolytic variants. The strains were isolated in one district and may be different representatives of the same strain, but this is uncertain.

Group A strains (28)					
Agglutinations	M precipitin tests	Colony forms	Amylase-positive strains	Proteolytic activity in cultures used for M typing	Inactive proteinase precursor in same culture
5, 27, 44	Type 5 7 strains	Matt translucent	–	–	+, ±, –
5, 27, 44	Non-precipitating 11 strains	Large opaque	11	–	+ + +, + +, +
5, 27, 44	Non-precipitating 7 strains	Variable opaque and glossy	–	–	±, –
12, 27, 44	Non-precipitating 3 strains	Mucoid (stable)	–	–	±, –

Factors possibly connected with loss of M antigen

The majority of amylolytic strains were isolated from patients with acute streptococcal infections and on the basis of clinical findings were certainly pathogenic. Three different Type 2 strains, for example, were associated with severe infections. It was possible that streptococci capable of elaborating M antigen *in vivo* were incapable of doing so outside the host, but there were several other possible factors to be considered. First there was the possibility that the M substance was destroyed by small amounts of proteinase in the same culture. Though amylolytic streptococci produced variable amounts of inactive proteinase precursor (Elliott & Dole, 1947) there was no evidence that proteinase was present in the cultures used for M typing. Another possibility was that the anti-M typing sera contained insufficient antibody because of the difficulty of making satisfactory sera for certain Types which was mentioned on p. 414. The M protein is poorly antigenic at best, and it might be that in the Types most often associated with amylase activity, it was even less effective as an antigen in the rabbit. This anomaly, however, suggested two other ideas, one being that the M antigen was lost from cells because it was all liberated into the culture medium.

According to Olarte (1948) certain Group A strains released reactive M protein in broth cultures. He also found free material in the same cultures which reacted with anti-T sera. Four strains used by Olarte were among the stock cultures tested for amylase (S23/94/2, J17E/100/6, J17F/38/1, D24/42/0) but none of them was amylase-positive. In a preliminary test broth cultures of strain J17/F (Type 26) contained protein which precipitated in unabsorbed homologous Type anti-M serum, and in anti-T serum, but which also reacted strongly with heterologous anti-M and anti-T sera of the closely related specific Types 4, 24, 28, 29 and 46. This suggested that the cultures contained free T substance, which is shared by these specific Types. The amylolytic strains were, however, different from those used by Olarte, because cellular M protein was not demonstrated.

Another possibility still remained, namely that the M protein was not lost from the cells in the sense that implies destruction, but that in these streptococci, it underwent a physico-chemical change which rendered it atypical. Early experiments made with a Type 2 strain (T2/44/Rb5) suggested that amylase was produced by certain matt-opaque colony variants found in blood-agar plate cultures and it seemed possible that the study of this phenomenon might furnish additional evidence about the relationship of amylase production to loss of M antigen.

Amylase production by variants

Besides the anomalies noted in connexion with serological typing of the amylolytic streptococci, it was found, as already noted, that about half the amylolytic strains generated mixed populations on starch agar, containing few or many amylase-positive colonies in a background growth of amylase-negative microcolonies. Before exposure to iodine enzyme-positive colonies were identifiable by their size and opacity, and also by faint halos of turbidity corresponding to the achroic zones revealed by iodine. Provided that the culture medium was clear and culture plates contained equal layers of agar there was rarely any difficulty in selecting amylase-positive variants for subculture.

For the study of variants, glutinous corn starch (Amioca) was a more sensitive substrate than ordinary starch. Like other bacterial amylases the streptococcal enzyme resembles α-amylase in that it rapidly degrades both amylopectin and glycogen, but has a relatively slight effect on amylose. Amioca contains more than 90 % of amylopectin, and it was therefore a more sensitive substrate than starch for the detection of weak activity. Following exposure to iodine the unchanged substrate was claret-coloured, nearer to the red brown of glycogen than to the purple of non-glutinous corn amylopectin.

The heterogeneity of amylolytic cultures was not appreciated until suitable broth dilutions (to 10^{-8}) were spread on substrate agar. Nearly all strains generated either greater or lesser numbers of enzyme-negative colonies ($<10\%$ to $>90\%$). When parent strains formed uniform growth in broth cultures, amylase-positive variants were distributed in decreasing numbers in higher dilutions, but in proportion to the numbers of amylase-positive variants in low dilutions. When strains formed granular broth cultures there were considerable discrepancies, the number of amylase-positive variants being disproportionately small in the higher dilutions. The streptococci deposited at single sites differed so widely in amylolytic power that the conclusion that amylase was produced by variants was inescapable. The same strains growing either on blood agar or plasma agar formed very large colonies in the uncrowded conditions of high dilution. In duplicate cultures on the substrate agar, certain strains gave rise to a few strong-positive variants, a few weak-positive variants, and a predominance of negative colonies. Single amylolytic colonies growing in relative isolation sometimes grew to great size (2 mm.) in relation to the rest of the population, but colonies which were producing small amounts of enzyme remained small in comparison, when growing in equally favourable conditions. The streptococci which produced the largest amounts of amylase were benefited by the presence of substrate, but the medium while supporting the growth of amylase-negative organisms, did not provide a favourable environment for exacting organisms such as Group A streptococci. The amylolytic property as a single character was first studied in serial subcultures on the same medium, and also in altered cultural conditions.

Transmissibility of the amylolytic property

Strong-positive amylolytic colonies were broken up in small volumes of Hartley digest broth and 0·2 ml. of the diluted cell suspension was spread on culture plates. Daily subcultures were made on substrate agar until weak-positive variants or negative colonies predominated in the populations or until the number of positive-variants was less than half the numbers found in the first subculture on starch agar. The results in Table 5 show that in some instances the amylolytic property was transmissible to the progeny of variants for as many as forty subcultures, amounting to a considerable number of generations. Other strains completely lost the property after one or two subcultures, and certain strains generated increasing numbers of weak-positive variants without entirely losing the ability to degrade starch.

The two most stable amylolytic strains among the sixteen shown in Table 4 were stock-cultures, T1/119/5 and T2/opaque, both received from Dr Lancefield. These were then propagated on 'subminimal' starch agar (prepared without Fildes's digest). In second or third subcultures, the populations contained 50% or more of enzyme-negative colonies but when the two strains were transferred to plasma agar for one or two subcultures, both recovered amylolytic power, which remained unimpaired for many generations in subcultures on the minimal medium. Weak-positive variants generated by strains T2/44/Rb5, 6068, C1010, O15104, RZ12 and RZ44 also completely recovered

Table 5. *The differences in stability of amylolytic variants generated by different Group A strains*

Group A strains	Precipitin tests M	Agglutination pattern	% of amylase positive colonies in primary culture	Nos. of subcultures in which approximately the same % of progeny were amylase positive
T1/119/5 (SF130/2)	Type 1	1, 2, 4, 28, 22	>90	46
SR707	Type 1	1, 2	>90	14
T2/44/Rb5 (SF22)	Type 2	2	>80	10
T2/Opaque	Type 2	2	>90	49
Brook	Type 2	2	80	8
6068	Type 2	2	55	6
C1010	Non-precipitating	4, 24	70	5
O15104	Non-precipitating	4, 24, 29	76	7
SR359	Non-precipitating	5, 27, 44	>90	10
RZ23	Non-precipitating	5, 12, 44	>90	5
RZ12	Type 25±	8, 25	90	15
RZ44	Non-precipitating	22, 14	85 (60 weak+)	4
DS21	Non-precipitating	22, 14	84 (20 weak+)	6
RZ90	Non-precipitating	22	30 (15 weak+)	4
SR1039	Non-precipitating	9	30 (20 weak+)	2
SR985	Non-precipitating	9	10 weak+	1

amylolytic power after one or two subcultures on plasma agar. The findings were reproducible when the procedure was repeated three or four times for each strain. The procedure was only partly successful in regenerating amylolytic power when applied to weak variants of certain other strains shown in Table 5 (SR1039, 985, RZ90). It failed when applied to fourteen Group A strains which were originally classified as doubtful amylase producers. Data relating to a total of fifty amylolytic strains indicated that when populations contained less than 30% of enzyme-positive colonies, the possibility of recovery on plasma agar was limited in comparison with that of strong positive strains. This suggested that certain strains lost the enzymic substance by dilution when grown on subminimal medium but elaborated more enzyme in enriched medium, and the capacity for recovery was a property inherent in the parent strain, and independent of substrate.

The substrate agar was then improved as a culture medium for Group A streptococci in general by addition of increasing amounts of Fildes's digest, crystalline haemin (5–10 μmg./ml. agar), or peptone (Neopeptone, Proteose peptone, Benger's peptone) and glucose (0·1–1%). Benger's peptone (2%, v/v) or Fildes's digest or haemin provided factors which promoted the recovery of amylolytic power by weak variants. Glucose concentrations greater than 0·3% promoted growth but inhibited amylase production. Duplicate cultures made on minimal and enriched substrate agar were incubated aerobically, anaerobically and in air containing 5% carbon dioxide, using six strains shown in Table 4: T1/119/5, T2/opaque, T2/44/Rb5, RZ12, C1010 and RZ90. All of the strains grew better either in anaerobic or carbon dioxide cultures than in air. Because the colonies were larger weak amylase production was more easily seen, but the same effect was obtained by prolonging aerobic incubation from 18 to 40 hr. at 37°. Thirty amylase-negative Group A strains were then re-tested for amylase production in varied cultural conditions. There was no evidence that the enrichment of the substrate agar induced the production of amylase by strains which were enzyme-negative on the minimal medium.

Amylase production by matt-colony and glossy-colony variants

Lancefield (1940) noted that among Group A streptococci colonial variation from matt to glossy forms was often associated with changes in antigenic structure. Matt-colony variants of certain strains usually contained M substance which was absent from or reduced in glossy variants. Certain amylolytic strains formed both matt and glossy colonies in plasma agar cultures, while other amylolytic strains growing on the same medium formed large-opaque and small-translucent colony variants. The large-opaque variants of several strains were designated intermediate, signifying a resemblance to the matt-form. When single-colony cultures were made on substrate agar, matt, intermediate and large-opaque variants often gave rise to numerous amylolytic colonies whilst glossy and small variants generated either few or no amylolytic organisms.

Six of the strains shown in Table 5 gave rise to distinct matt-colony and glossy-colony variants: T1/119/5, T2/opaque, RZ23, 6068, Brook and T2/44/Rb5. The antigens of the last two strains were studied by Maxted (1953) who isolated matt and glossy cultures with and without M antigen. The other four strains were designated 'matt' when first received in the laboratory and glossy-colony variants were isolated from subcultures. Cell suspensions of different colony variants, diluted to contain approximately equal numbers of organisms were seeded on substrate agar plates in serial dilutions for enumeration of amylolytic progeny.

Matt colony cultures were strongly amylase positive; glossy colony cultures were amylase negative, or weakly positive in one instance. Matt strains were then repeatedly subcultured on plasma agar, selecting colony variants which were identical with the parent matt form, and also colonies which differed from the parent in size, opacity or glossiness. Loss of amylolytic power was associated with change of colony form, namely the matt-to-glossy variation, but certain strains gave rise to intermediate and small opaque colonies which showed weakened amylolytic power in comparison with matt variants. Thirty-six amylolytic strains (of which twenty-seven were untypable by M precipitin tests) showed the same phenomenon, namely the association of amylase production with matt-opaque, large opaque and intermediate colony variants, and the weakening or loss of the property in glossy or near to glossy variants.

Properties of matt-colony cultures in relation to amylase production

At intervals during subculture matt and glossy strains were tested for Group A polysaccharide, for agglutinating antigens, for proteinase and for its inactive precursor. Cell extracts were prepared (1) by digesting organisms with trypsin and pepsin, (2) by heating organisms for different time intervals at pH 2, either at 37° or 57°. Matt strains produced larger amounts of the inactive proteinase precursor than glossy strains, and this difference was greater when matt strains were grown in media containing starch. Since these strains produced more amylase, the fermentation of starch was accompanied by a fall in pH value, and this produced conditions suitable for the release of the precursor (Elliott, 1950). Matt strains, however, also contained large amounts of a cell substance extractable by crystalline trypsin (0·01 mg./ml. culture for 6 hr., at 37°, at pH 8·0) which precipitated in homologous Type anti-M serum, and in some instances with certain heterologous Type anti-M and anti-T sera. This substance, which was not successfully extracted by pepsin, also precipitated strongly in proteinase precursor anti-serum, prepared with crystalline precursor and absorbed with crystalline proteinase. Elliott (1950) found that the precursor anti-serum did not react with other streptococcal products, though crystalline precursor reacted with anti-serum prepared with partially purified deoxyribonuclease. The trypsin-extractable substance therefore contained at least two substances, namely the precursor and the material reacting with the type-sera. It also contained amylase. Since glossy strains contained relatively small amounts of the trypsin-extractable substance and produced only small amounts of amylase, it seemed possible that this cell component was associated with the amylolytic power of the matt variants.

The serological activity of the soluble substance was similar to or identical with the agglutination pattern of the strain recorded during slide-agglutination tests. The strain T1/119/5 (SF130), for example, was agglutinated by antisera for Types 1, 2, 4, 28 and 22, and the material extracted with trypsin precipitated in homologous Type 1 unabsorbed anti-M and anti-T sera, and also in heterologous Type sera, 2, 4 and 28. The strain RZ44, shown in Table 5, was identified by slide agglutination as Type 22, but showed cross-reactions with Type 14. Cell extracts reacted with Type 14 unabsorbed anti-M sera, and also with anti-T sera Types 14 and 22. The material extracted by trypsin during 6 hr. was presumably resistant to digestion by trypsin and was therefore like T antigen. However, the yield of reactive material obtained by digesting the organism with pepsin was negligible in comparison, which was unlike T antigen (Lancefield & Dole, 1946). When 6 hr. extracts (pH 7·8), still containing trypsin, were freed from cells and the filtrates were re-incubated overnight at 37°, the reactivity with proteinase precursor antiserum was not significantly altered, but both amylase activity and reactivity with Type sera were destroyed. The free substance was therefore trypsin sensitive, which was also unlike T antigen. Slow inactivation took place at bench temperature or at 37° in the absence of trypsin. When cell-free extracts were re-incubated at 37° with 1 % glutinous corn starch the reactivity with homologous Type anti-M and anti-T sera was not destroyed.

Trypsin-extractable substance from strain RZ44, Type 22

Organisms were harvested from 3·5 l. of 24 hr. broth culture (Neopeptone infusion broth) of strain RZ44. The cells were washed 4 times in buffer, and the centrifuged sediment suspended in buffer pH 7·8 containing crystalline trypsin 0·01 mg./ml. The suspension was incubated for 6 hr. at 37°. The supernatant was made cell-free by filtration. The crude extract (100 ml.) reacted with Type 14 anti-M serum and proteinase precursor anti-serum to a titre of 1/64 (antigen diluted). The Type 14 serum had been previously absorbed with an M extract of heterologous Type.

The reactive protein was soluble in slightly acidified 70% ethanol but precipitated by 80% ethanol at pH 7·4 or higher pH values, therefore it was further purified by fractional precipitation with cold ethanol discarding substances insoluble in 25, 50 and 70% ethanol in the pH range 7·0–5·6. The reactive protein then precipitated from the supernatant by 80% ethanol at pH 8·0 was part crystalline and part amorphous, and was further purified by reprecipitation at pH 2·5 following the method used by Lancefield & Dole (1946) to purify T antigen. After five precipitations, the protein crystallized in 90% ethanol at pH 8, and was then crystallized six times in ethanol. The final product precipitated in Type 14 anti-M serum and proteinase precursor antiserum; the results of precipitin tests are shown in Table 6. A single absorption with the purified antigen removed the precipitating antibody from the Type 14 anti-M and precursor antisera, but did not remove either the M antibody from the one, or the precursor antibody from the other.

The strain RZ44 liberated large amounts of a similar reactive protein into culture media containing substrate, which, after purification and crystallization by the same procedure was serologically identical with the cell substance. The purified free protein was antigenic in the rabbit. The immune serum precipitated the homologous free antigen, the homologous cell antigen, and the crystalline proteinase precursor. A single absorption either with the free antigen or the cell antigen removed the precipitating antibody, which was not however, completely removed by absorption with the crystalline precursor. The purified antigens contained amylase but it is still uncertain if the enzyme is identical with, part of, or derived from the antigen.

DISCUSSION

Among haemolytic streptococci of human origin, amylase-producing strains have at present been found only among members of Group A. Trypsin-resistant cell antigens related to Group A serological Types have been found in Groups B, C and G strains, namely the R antigen of Type 28 (Lancefield & Perlman, 1952) and the T antigen of Type 2 (Maxted, 1953). Hyaluronidase-producing Groups C and G strains which were specifically agglutinated by Group A antisera for Types 2, 5, 25 and 28 were found by Crowley (1944). The Type 22 antigen, which with Type 4 is associated with hyaluronidase activity in Group A, has not yet been reported in connexion with other serological groups. Serological Types, therefore, which are associated with

Table 6. *Serological activity of purified cell antigen (trypsin-extractable) from strain RZ44*

The partially purified cell antigen RZ44, precipitated in heterologous anti-M and anti-T sera (1/1 dilution of antigen, + or ±) for Types 22, 9 and 18. The cell antigen, obtained by extraction with trypsin, was purified by ethanol fractionation and precipitation at pH 2·5. The free antigen (crystalline FA) used to prepare immune serum was isolated from culture medium and purified by same procedure. Concentration of crystalline proteinase precursor = 0·15 mg./ml. Cell antigen RZ44 and precursor solutions had approximately equal amounts of protein (spectrophotometric 280 μ.

		Purified cell antigen, RZ44 antigen diluted								
Antisera	Absorption	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
RZ44, crystalline FA	.	++++	+++	+++	+++	+++	+++	+++	++	+
	Free antigen RZ44	–	–	–	–	–	–	–	–	–
	Cell antigen RZ44	–	–	–	–	–	–	–	–	–
Inactive proteinase precursor (previously absorbed with crystalline proteinase)	.	+++	++	++	++	+	±	–	–	–
	Free antigen RZ44	–	–	–	–	–	–	–	–	–
	Cell antigen RZ44	–	–	–	–	–	–	–	–	–
Type 14, anti-M	.	++++	+++	+++	+	±	±	–	–	–
	M antigen Type 6	++++	++	+	–	–	–	–	–	–
	M antigen Type 6 and cell antigen RZ44	–	–	–	–	–	–	–	–	–
Normal rabbit serum		±	–	+	–	–	–	–	–	–

		Crystalline proteinase precursor antigen diluted								
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
RZ44, crystalline FA	.	++++	++++	+++	+++	+++	+++	+++	++	+
	Free antigen RZ44	–	–	–	–	–	–	–	–	–
	Cell antigen RZ44	–	–	–	–	–	–	–	–	–
Inactive proteinase precursor (previously absorbed with crystalline proteinase)	.	++	++++	++	+	+	±	–	–	–
	Free antigen RZ44	–	–	–	–	–	–	–	–	–
	Cell antigen RZ44	–	–	–	–	–	–	–	–	–
Type 14, anti-M	.	±	–	–	–	–	–	–	–	–
	M antigen Type 6	–	–	–	–	–	–	–	–	–
	M antigen Type 6 and cell antigen RZ44	–	–	–	–	–	–	–	–	–
Normal rabbit serum		–	–	–	–	–	–	–	–	–

amylolysis in Group A, are also associated with phenomena in Groups C and G, namely the presence of trypsin-resistant cell antigens and the production of hyaluronidase. There was, however, no strict correlation between the presence of the cell antigen and the ability to elaborate the enzymes. If the amylase is a Group A specific substance, which requires further confirmation, it may be possible that the presence of certain T- or R-like factors may confer trypsin-resistance on a cellular component derived from or related to the M antigen. The purified proteins in the instance described had physical and chemical properties similar to those of M antigen, namely alcohol-solubility, heat-resistance and trypsin-sensitivity. The cell antigen was however extracted by trypsin, following the method used to extract R antigen and, though unlike T antigen it was not extractable by pepsin, the substance was purified by the method used by Lancefield & Dole (1946) to purify T antigen.

At present the amylolytic substance cannot be equated with M, T or R antigen on the basis of physico-chemical or immunological properties. The interpretation of the serological findings depends on the purity of the antigens and the antibody content of the sera. Elliott (1950) suggested that the inactive proteinase precursor may be part of a larger molecule. It seems possible that the amylase and the precursor may be released from the cell as part of such a molecule derived from, or related to, the M and T antigens of the present strains.

It was pointed out by Stanier (1953) that a phenomenic property singled out for study as a character may be an incidental by-product conferring other essential properties on the organism. Amylolytic strains are enabled to survive in an environment unfavourable for Group A strains in general; the by-product confers this essential property on the organisms. The ability to confer stable amylolytic power on the progeny was associated with the possession of a cell antigen, and with the ability of the present strain to remain matt or near to matt. The maintenance of this phase may depend on the degree of resistance of the cell antigen, to proteolytic digestion. This may also be a factor regulating the rate of its release from the cell.

I wish to thank the Director and staff of the Streptococcal Reference Laboratory for supplying the majority of the strains.

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(*Received* 28 *October* 1953)

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Evidence for Two Forms of Staphylococcal Coagulase

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SUMMARY: Staphylococci produce coagulase in two forms. One is bound to the cell wall and is responsible for the slide test. The other is liberated as free coagulase in the medium and is responsible for the tube test. Bound coagulase acts directly on the fibrinogen of certain animals which then causes clumping of the staphylococcal cells, while free coagulase acts on the 'prothrombin' of certain animals to give a thrombin-like product. Bound coagulase is antigenically distinct from free coagulase. It is released during autolysis and it can be used to produce specific antibodies in rabbits.

Two tests are now in use for the detection of staphylococcal coagulase in cultures. In the tube test, first described by Loeb (1903), the bacteria are grown in a sensitive plasma, which is usually diluted, for periods up to 24 hr.; coagulase production is then shown by the presence of a clot. A later development is the slide test (Much, 1908; Birch-Hirschfeld, 1934), which depends on the finding that when coagulase-positive staphylococci are emulsified in a sensitive plasma on a slide, thick clumps appear due to precipitation of the fibrinogen around the bacteria.

Cadness-Graves, Williams, Harper & Miles (1943) made a large-scale comparison of the two tests and found a good correlation between them. Of 442 strains recovered from pathogenic conditions in humans, all but two gave both reactions. A further series obtained from wounds and nasal swabs showed more disagreement in that forty-eight strains were tube positive and slide negative, while three were tube negative and slide positive. Following this series, it was commonly assumed (Berger, 1943) that the two tests were measuring the same substance. Observations by Linsell & Gorill (1951) threw some doubt on this assumption. They showed that plasma treated by heat or thiomersalate continued to give a positive slide test while losing its ability to give a positive tube test; no explanation was offered. In analysing the effect of heat on plasma it became clear that two coagulases were involved, one bound to the cells and giving the slide test, and one free in the medium and giving the tube test. These will now be referred to as bound and free coagulase, and evidence for their distinction will be presented.

MATERIALS AND METHODS

Free coagulase. The free coagulase was a lyophil dried preparation (16,000 M.C.D./mg.) from the 'Newman' strain of *Staphylococcus aureus* (Duthie & Lorenz, 1952), and was normally used at a concentration of 0·5 or 1·0 mg./ml. Tests were made by adding 0·2 ml. to 0·2 ml. of a suitable coagulable material in tubes 5·0 × 0·8 cm. The tubes were held against a black background in a warm illuminated box, and tapped until a clot appeared, the time being measured. For times up to 3 min. the reciprocal of the coagulase concentration plotted against the clotting time gave a straight line (Duthie, 1954).

Bound coagulase. Unless otherwise stated, the strain used was 'Newman', previously described as a good producer of free coagulase (Duthie & Lorenz, 1952). The cells from shaken digest broth cultures were washed in saline and resuspended to a standard density (4×10^9 cells/ml.); 0·2 ml. of this suspension was added to 0·2 ml. of diluted plasma or to 0·2 % bovine fibrinogen in $5{\cdot}0 \times 0{\cdot}8$ cm. tubes and shaken in a special rack in a Kahn shaker. Against a black background using direct lighting and a lens, the degree of clumping could be graded quantitatively and was recorded as −, tr., ±, +, +± or ++.

Titration of antibody to bound coagulase and of inhibitor to this antibody. For antibody titration 0·1 ml. volumes of falling dilutions of antibody in saline were added to 0·1 ml. of a standard 'Newman' suspension and left for 30 min. on the bench. One-tenth ml. of 0·2 % bovine fibrinogen was added, and after shaking in a Kahn shaker until the controls became positive (1–2 min.), the tubes were read as in the titration of bound coagulase. In the antibody inhibition test 0·1 ml. volumes of falling dilutions of antiserum (Wellcome Research Laboratories staphylococcal antiserum KCP 2029), usually in dilutions of 1/160 to 1/500, were added to 0·1 ml. volumes of the solutions to be tested for inhibitor. At the same time a control series containing saline instead of inhibitor was set up. The mixture was left for 30 min. on the bench, 0·1 ml. of the standard suspension was then added and after mixing left for another 30 min.; 0·1 ml. of bovine fibrinogen 0·2 % (w/v) was then added, and after shaking until the control tubes were positive at 1/500 antiserum, the results were recorded as before.

Activator. This was measured by adding 0·1 ml. solution of free coagulase to a mixture of 0·1 ml. activator solution and 0·2 ml. of 0·4 % (w/v) bovine fibrinogen (Armour's Fraction I). Since clotting times plotted against the reciprocal of the activator dilution gave a straight line over a limited range, comparisons were possible.

Purified prothrombin. This was prepared from human plasma following the method of Jaques (1943). It was used at a strength of 1·0 mg./ml.

Crystalline trypsin. This contained approximately 50 % $MgSO_4$, and was that used by Duthie & Lorenz (1949), when it was assigned the arbitrary value of 20,000 units/mg.

Differences between bound and free coagulase

Effect of heat on plasma. The first indication of a difference between the two types of coagulase was given by the behaviour of heated plasma. Human or rabbit plasma diluted to 10 % (v/v) in saline and heated at 56° for 4 min. no longer clotted with free coagulase or with thrombin. Failure to clot with free coagulase was not due to loss of activator which was always *c.* 50 % of the original. On the other hand, this heated plasma readily clumped the cells in the bound coagulase test, though more slowly and less strongly than did unheated plasma. Plasma brought to pH 2 and then neutralized behaved in the same way as heated plasma.

Mode of action of the two coagulases. As first shown by Smith & Hale (1944), free coagulase will only clot guinea-pig plasma in the presence of an activator found in the plasmas of certain animals. Duthie & Lorenz (1952) produced evidence to show that the activator is probably prothrombin; a typical experiment is shown in Table 1.

Table 1. *Experiment to show that the action of free coagulase depends on the presence of a sensitive prothrombin or a closely related substance*

All dilutions were made in saline.

0·2 ml. free coagulase + 0·2 ml. guinea-pig plasma 10 % (v/v)	No clot
0·2 ml. free coagulase + 0·2 ml. guinea-pig plasma 10 % (v/v) + 0·1 ml. human prothrombin purified	Rapid clot
0·2 ml. free coagulase + 0·1 ml. human prothrombin purified	No clot
0·2 ml. free coagulase + 0·2 ml. guinea-pig plasma 10 % (v/v) + 0·2 ml. of 0·2 % (w/v) bovine fibrinogen	No clot

Table 2. *Experiment to show that the action of bound coagulase depends on the presence of a sensitive fibrinogen and not on an active prothrombin*

(All dilutions made in saline.)

0·2 ml. washed cells + 0·2 ml. guinea-pig plasma 10 % (v/v)	Nil
0·2 ml. washed cells + 0·2 ml. guinea-pig plasma 10 % (v/v) + 0·1 ml. heated human prothrombin	Nil
0·2 ml. washed cells + 0·2 ml. guinea-pig plasma 10 % (v/v) + 0·1 ml. of 0·02 % bovine fibrinogen	Clump
0·2 ml. washed cells + 0·2 ml. saline + 0·1 ml. of 0·02 % (w/v) bovine fibrinogen	Clump

By contrast washed cells representing bound coagulase will not clump in guinea-pig plasma, even when human prothrombin is added (Table 2). The purified prothrombin in this case was heated for 4 min. at 60° to denature small traces of human fibrinogen which are difficult to remove. The loss of activator by heating was again *c.* 50 %. Since the addition of a minute amount of bovine fibrinogen, which contained no activator, was sufficient to cause clumping there was no evidence of inhibitory action by the guinea-pig plasma. Similar results were obtained when freshly precipitated guinea-pig fibrinogen was used instead of guinea-pig plasma.

Species specificity of plasmas to the two coagulases. Species specificity of plasma to the action of bacterial toxins is well known in the case of streptokinase and free coagulase. It was therefore not surprising to find that certain plasmas, e.g. sheep and guinea-pig, were completely insusceptible to the action of bound coagulase, others, for example, cow and horse, occupied an intermediate position, while dog, mouse, human and rabbit plasmas were highly sensitive. Species differences for bound coagulase were best measured by finding the limiting dilution of plasma (plasma titre) sufficient, on prolonged shaking, to clump a given suspension of staphylococci when a highly active strain such as 'Newman' was used (Table 3). This was a measure of the sensitivity of the fibrinogen to bound coagulase and did not compare with the findings for free coagulase which depended on the sensitivity of the activator (prothrombin). Thus, of the eight plasmas listed only three, e.g. horse, human and rabbit, were good sources of activator and clotted readily with free coagulase, while all but guinea-pig and sheep contained fibrinogen which reacted readily with bound coagulase.

Table 3. *Comparison of plasmas of different animals as a source of activator for free coagulase and of fibrinogen for bound coagulase*

Activator test mixture was 0·1 ml. of 0·1 % (w/v) free coagulase +0·2 ml. of 0·4 % (w/v) bovine fibrinogen+0·1 ml. of 10 % plasma. The plasma titre was that dilution of plasma causing perceptible clumping of the cell suspension on 10 min. shaking.

Plasma	Clotting time using plasma as activator for free coagulase	Plasma titre for bound coagulase
Sheep	>10 min.	Nil
Guinea-pig	>10 min.	Nil
Cow	>10 min.	1/100
Horse	2 min.	1/50
Dog	>10 min.	1/2000
Mouse	>10 min.	1/1000
Human	50 sec.	1/2000
Rabbit	40 sec.	1/2000

Strain differences in clumping with fibrinogen. Strains of staphylococci differed very much in their ability to clump in the presence of fibrinogen, and a rough measure was the limiting dilution of human plasma just sufficient to cause clumping. About twelve strains from various sources were compared, and it was found that they varied widely between those which gave titres of 1/30 for poor strains and two strains which gave good clumping at final dilutions of 1/4000 of human plasma, corresponding to a final dilution of one part in three millions of fibrinogen. The two strains which gave these high titres were the best strains so far encountered for the production of free coagulase in the absence of albumin (Duthie, 1954). Later work showed that the plasma titre for clumping bore no relation to the amount of bound coagulase present on the cell surface. The phenomenon is under investigation.

Like free coagulase, bound coagulase was stable at pH 2. The washed and acid-treated cells gave an even higher plasma titre than before, but this may have been due in part to their tendency to show some slight clumping in saline only. Living cells treated with crystalline trypsin rapidly lost their bound coagulase.

Apparent dissociation of the production of the two types of coagulase has been found in several strains isolated from routine nasal swabs, inasmuch as they produced large amounts of free coagulase in the presence of albumin but failed to clump when added to plasma or fibrinogen and shaken. This confirms the findings of Cadness-Graves *et al.* (1943). The converse has not yet been encountered.

Immunological specificity of the two coagulases. Antibodies to free coagulase have been found in the sera of normal humans (Lominski & Roberts, 1946; Bekker, 1947; Tager & Hales, 1948*b*; Rammelkamp, Hezebicks & Dingle, 1950; Duthie & Lorenz, 1952). They have been produced experimentally in animals (Tager & Hales, 1948*a*; Rammelkamp, Badger, Dingle, Feller & Hodges, 1949; Duthie & Lorenz, 1952). These antibodies are specific for the free coagulase of different staphylococcal strains (Duthie, 1952). None of the antisera against free coagulase had any inhibitory action on bound coagulase. Certain commercial antisera made against the Wood 46 α-lysin inhibited bound but not free coagulase. The most satisfactory of these was a horse antiserum KCP 2029 made at the Wellcome Research Laboratories, Beckenham, and containing 400 u./ml. of anti α-lysin. Diluted 1/500 this usually gave partial inhibition in the antibody test using the standard bound coagulase suspension (staphylococcal strain 'Newman') and complete inhibition at higher levels. A number of commercial non-staphylococcal equine antisera examined contained no antibody; small amounts were present in normal human sera and these will be investigated when the test is fully standardized. No antibody was found in normal animal sera, e.g. mouse, guinea-pig, rabbit, horse, sheep and cow.

Identification and partial purification of bound coagulase liberated by autolysis

Antibody inhibition test. Since the sera of horses immunized with staphylococcal toxoid made from cell-free filtrates of the Wood 46 strain contained an antibody to bound coagulase, it was likely that bound coagulase was liberated from these cells. Using the antibody inhibition test, described earlier, as a test for the presence of bound coagulase in cell-free culture material, no inhibitor was found in Wood 46 toxin or toxoid, or in broth cultures of several freshly grown staphylococcal strains. An inhibitor (later shown to be bound coagulase) was found in the supernatants of shaken broth cultures of coagulase-positive staphylococci which had been stored for several weeks at 4° and which had undergone partial autolysis. The inhibitor was more easily detected if the cell-free supernatants were dialysed. Maximum amounts were present in supernatants of the 'Newman' strain, and a study of freshly grown cultures of this organism showed an increase when the flasks were left at room temperature. This was not associated with a gain in free coagulase (Table 4). The best yields were from cultures held between pH 6 and 8.

Partial purification of bound coagulase. Since the inhibitory material was stable at pH 2 it was easily concentrated and partially purified by precipitation with $CdSO_4$ between pH 6 and 7, and the precipitate redissolved by the addition of HCl and dialysed against 0·01 M-HCl. Such precipitates usually contained large amounts of free coagulase. In one culture which had undergone autolysis over a period of several months (Table 4) almost all the free coagulase, together with the remaining cells, was first removed by centrifuging at pH 3. More recently, the autolysis of washed cells at 4° has provided material with good inhibitory power and which contained little or no free coagulase.

Properties of partially purified bound coagulase. Like free coagulase purified inhibitor was most stable in the acid range, and least in the alkaline (Table 5). It was also destroyed completely by crystalline trypsin, though not in the

Table 4. *Effect of ageing of broth cultures of staphylococcus strain 'Newman' on liberation of an inhibitor, presumably bound coagulase, to bound coagulase antibody.*

Cultures were grown to about $0{\cdot}5 \times 10^{10}$ organisms/ml. left at rest, freed from cells, dialysed and tested at two-fold dilutions.

	Antiserum to bound coagulase				Free coagulase (u./ml.)
Age of culture	1/260	1/400	1/500	0	
4 months, 4°	+	+	+	++	< 100
4 days, 20°	–	±	++	++	3000
2 days, 20°	–	±	+	++	3000
12 hr., 20°	–	–	tr.	++	5000
Broth control tube	–	–	±	++	–

++ = gross clumps; + = moderate clumps; ± = faint clumps; tr. = very faint clumps; – = no clumps.

presence of soya-bean trypsin inhibitor. In these respects it corresponded to bound coagulase when attached to the cells. The appearance of inhibitor in the broth supernatants of fully grown cultures left to autolyse was accompanied by a loss of bound coagulase from the cell surface. There was therefore strong presumptive evidence for regarding the inhibitory material as bound coagulase which had been liberated from the cell surface. The addition of this material when partially purified to purified human or bovine fibrinogen, caused no clotting in 1 hr., nor was there any evidence of digestion of the fibrinogen.

Table 5. *Stability of bound coagulase (inhibitor) after liberation from staphylococcal cells*

	Dilutions of antiserum				
	1/160	1/200	1/260	1/400	Saline
	Effect of suspension of staphylococci				
1. Original inhibitor solution	±	+	++	++	++
2. After 48 hr. pH 2 room temp.	+	+±	++	++	++
3. After 48 hr. pH 7 room temp.	±	+	++	++	++
4. After 48 hr. pH 9 room temp.	tr.	±	+	++	++
5. Inhibitor + trypsin held 1 hr. pH 9 37°, then boiled	–	–	–	±	++
6. Inhibitor + trypsin first boiled; then held 1 hr. pH 9 37°	±	+	++	++	++
7. Control: no inhibitor added	–	–	–	±	++

++, ± = gross clumps; + = moderate clumps; ± = faint clumps; tr. = very faint clumps; – = no clumps.

Activity measured by its ability to inhibit antiserum to bound coagulase. Final concentration of trypsin 2×10^{-4} (w/v).

Experimental antibody production. The supernatant broth from a culture of the 'Newman' strain which contained inhibitor was dialysed and boiled for 10 min.; this inhibitor was adsorbed almost completely by the addition of 2 mg. sterile $AlPO_4$/ml. A comparison was made of the immunizing properties of the adsorbed and unadsorbed material (Table 6). Normal rabbit sera showed no inhibition of bound coagulase. After three injections at weekly intervals, each consisting of adsorbed material from 3 ml. of the original broth, one rabbit serum showed partial inhibition at a titre of 1/32 and the other at 1/16. Very much lower titres were obtained with culture fluid which had not been adsorbed on $AlPO_4$ (Table 6). It was concluded that the supernatants of stored 'Newman' strain cultures contained bound coagulase, which had been liberated during autolysis.

Table 6. *Production in rabbits of antibody to bound coagulase by use of the inhibitory material of Table* 5 *either unadsorbed or adsorbed on* $AlPO_4$. *Injections given on* 1*st*, 7*th and* 14*th days of the experiment*

			Day 1, undiluted serum	Day 28, serum dilution				
Dosage				2	4	8	16	32
			Effect of suspension of staphylococci					
Rabbit	$AlPO_4$	Supernatant						
23	7 mg.	3 ml.	++	−	−	−	−	+
26	7 mg.	3 ml.	++	−	−	±	+	++
32*a*	Nil	1st day, 1 ml. 7th day, 2 ml. 14th day, 3 ml.	++	±	++	++	++	++
3 *b*	Nil		++	−	±	++	++	++
Control	0	0	++	++	++	++	++	++

++ = gross clumps; + = moderate clumps; ± = faint clumps; − = no clumps.

Comparison of the slide and tube tests

A comparison of the slide test with the test used in the present work for bound coagulase, under varied conditions, showed no discrepancies. The slide test is therefore due to bound preformed coagulase on the cells. Under the conditions used by Linsell & Gorrill (1951) this acts on plasma treated by heat or with thiomersalate and causes clumping of the cells.

By contrast the tube test requires the production of a clot, which is a function of free coagulase. Experiments have shown that even with a good coagulase-producing strain of staphylococci, insufficient free coagulase would be carried over in the inoculum used by Linsell & Gorrill (1951); hence a positive result must be due to the production of free coagulase during the 2 hr. incubation period. This was of course impossible in the presence of 1/10,000 thiomersalate; the cells failed to grow and the test was negative. Tests of plasma treated with 1/10,000 thiomersalate for 24 hr. have shown no loss of activator, and a minor change in the fibrinogen as indicated by a slight delay in the reaction when treated with bound coagulase, free coagulase or thrombin.

DISCUSSION

Proof of the separate identities of bound and free staphylococcal coagulase falls under three main headings; of these, the most important is mode of action. Free coagulase apparently acts on prothrombin or on some closely related substance and converts it to a thrombin-like product which will clot any fibrinogen. Bound coagulase, on the contrary, acts on a susceptible fibrinogen and alters it so that it precipitates on the staphylococci and causes them to clump. Both substances behave as proteins; free coagulase may be an enzyme since the conversion of prothrombin to a thrombin-like product by free coagulase follows an enzymic pattern (Miale, 1949; Duthie & Lorenz, 1952). With bound coagulase prior incubation of the washed cells with minimal amounts of fibrinogen showed no shortening of the clumping time when the cells were agitated. Likewise, washed cells mixed with fibrinogen and removed immediately by centrifuging were just as strongly agglutinated as those left in contact with the fibrinogen for a longer time. The reaction may therefore be a stoichiometric one.

The second main point of difference lies in their different antigenic compositions; for many immunologists this would be conclusive. There is no known case of the same bacterial cell producing the same toxin in two different antigenic forms. Yet rabbit antisera against free coagulase failed to neutralize the bound coagulase on the cells from which the free coagulase had been obtained, and the antiserum KCP 2029 had no inhibitory action on free coagulase. Although antibodies to free coagulase are specific for the coagulase of certain staphylococcal strains, animal or human, present evidence suggests that the antibody for bound coagulase is non-specific.

The last point concerns the nature of the antibody inhibitor found in partially lysed broth cultures. The evidence so far accumulated suggests that this is originally bound coagulase which becomes liberated during autolysis. The strongest evidence for this is its ability to produce in rabbits antibody to bound coagulase. While the maximum amount of free coagulase was present at the end of growth, this inhibitory material did not appear in any quantity for several days. Separation of the two was possible in one culture tested by precipitation of the free coagulase and its removal with the cells by centrifuging at pH 3 leaving the inhibitor free in the medium, or by autolysing washed cells. Like free coagulase it was stable in the acid pH range, and was partially purified by precipitation with cadmium, followed by dialysis in acid. This partially purified material had apparently no immediate action on fibrinogen when incubated with it; it is being further examined.

Besides Linsell & Gorrill (1951) other authors have noted discrepancies in the behaviour of staphylococci as between slide and tube tests. Birch-Hirschfeld (1934) concluded that the slide test was caused by an agglutination of staphylococci by prothrombin and that the tube test was caused by a clotting of fibrinogen. Her conclusions are open to doubt, inasmuch as the fibrinogen solutions used contained prothrombin and the prothrombin solutions almost certainly contained denatured fibrinogen. In a survey of the slide test using plasmas and strains derived from different animals, Field & Smith (1945) noted that negative slide tests were given by sheep and guinea-pig plasmas, in contrast to other plasmas which gave positive reactions. The addition of human sera to these insensitive plasmas failed to make them positive, as it should have done had the reaction been similar to that of the tube test. No attempt was made to explain the discrepancy between the two tests.

There are several references in the literature to an agglutination of streptococci by sera from patients suffering from acute bacterial infections (Weaver, 1904; Tillett & Abernethy, 1932). The reaction did not appear to be caused by specific antibodies but to a non-clottable protein which resembled fibrinogen (Tillett & Garner, 1934). The latter authors showed that such strains of streptococci, when mixed with plasma or fibrinogen to dilutions as high as 1/40,000–1/80,000 and incubated for 2 hr. at 37°, showed clumping when allowed to settle in the cold. There is thus a strong probability that streptococci produce a substance similar to the bound coagulase of staphylococci, and that the material in the sera of patients is a partly denatured fibrinogen similar to that obtained in the present study by acid or heat treatment of plasma.

From the survey of Cadness-Graves *et al.* (1943) it is clear that all pathogenic strains of staphylococci form free and bound coagulase. Strains which apparently produce only free coagulase are found occasionally in nasal and throat swabs and are of doubtful pathogenicity. As a consequence it would seem that the slide test alone is an adequate test for pathogenicity, and where there is doubt the organisms may be suspended in human plasma and shaken to see whether clumping occurs, a comparable saline suspension being used as a control. The tubes used in the present experiment are ideal for this purpose. The importance of these two coagulases in staphylococcal infections has yet to be determined. Hale & Smith (1945) showed that free coagulase can produce *in vitro* a network of fibrin around leucocytes and thereby produce a mechanical obstacle to phagocytosis; they suggested that the same process may occur *in vivo*. There is as yet no proof that this happens in infections though Smith, Hale & Smith (1947) reported greater virulence of staphylococci when injected in plasma which was coagulable by these bacteria. Rogers & Tompsett (1952) showed that coagulase-negative staphylococci were destroyed following ingestion by phagocytes, but coagulase-positive strains survived and killed the leucocytes. This might be due to the ability of these strains to produce α-lysin, but it is tempting to speculate that the presence of bound coagulase on the cell surface may hinder their being digested. A comparison of the survival of strains which produce α-lysin or bound coagulase, but not both, might settle this point.*

I am grateful to Dr D. W. Henderson, Director of the Microbiological Research Department (Ministry of Supply), Porton, Wiltshire, for making available the facilities of his department for the production of coagulase. I wish to thank Dr R. A. Kekwick of the Lister Institute, London, for providing the specimens of fibrinogen and for very helpful advice, and the Wellcome Research Laboratories, Beckenham, Kent, for supplies of antiserum. I am also grateful to Miss J. Rowlands and Miss S. Isherwood for valuable technical assistance. The work was financed by a research grant from the South-West Metropolitan Regional Hospital Board.

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* The author now considers that the term 'clumping factor' is better than 'bound coagulase'.

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(*Received* 13 *November* 1953)

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The Production of Free Staphylococcal Coagulase

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SUMMARY: Free staphylococcal coagulase is formed early in the lag phase and is released continuously, mainly at the first division in heavily seeded cultures. With most strains the presence of serum albumin in the medium greatly enhances the production of both free and bound coagulase (Duthie, 1954). The albumin factor is relatively heat stable, but is easily destroyed by crystalline trypsin.

Duthie (1954) has shown that there are at least two forms of staphylococcal coagulase, one bound and one free, which differ in their mode of action, antigenic pattern and occurrence. Previous studies on coagulase production were concerned only with free coagulase which is liberated into the medium and is recognized by the formation of a clot when added to a suitable plasma. Walston (1935), Walker, Derow & Schaffer (1948) and Tager & Hales (1947) produced free coagulase in liquid media such as digest broth, when the bacteria were grown in static culture at 37° for periods up to a week. Lominski (1944) showed that coagulase production under these conditions was enhanced by the addition of human plasma to the medium. Davies (1951) studied the effect of serum on the production of coagulase when large inocula were used. He stated that coagulase was produced only during the latter part of the lag phase, and that the addition of rabbit serum at this time to a broth initially without serum produced a rapid release of coagulase into the medium. In studies by Duthie & Lorenz (1952), who used only one strain ('Newman') of staphylococcus, it was repeatedly shown that addition of plasma or serum to the medium did not increase the yield of free coagulase. On repeating the experiments of Davies with other staphylococcal strains it was found that most of them gave increased yields of coagulase in the presence of serum. However, of the four strains used to produce coagulase of the four different antigenic types (Duthie, 1952), three (including strain 'Newman') did not show enhancement and consequently were atypical. So far it has not been possible to produce the coagulase of the fourth one, type D, in any quantity without the addition of serum or albumin to the medium. In consequence it has not been possible to obtain it in a form suitable for a vaccine. The studies now reported were incidental to solving this problem, and though this has not been achieved, they have revealed some new factors concerning coagulase production.

METHODS

Coagulase production. Stock cultures of staphylococci for seeding were obtained by growing strains in digest broth in flasks supported on a slightly inclined rotating gramophone turntable. The total count was $4\text{–}10 \times 10^9$ organisms/ml. at 18 hr. and cultures could be stored for a week at 4° without alteration in their behaviour when used for seeding. Unless otherwise stated, stock cultures were diluted to 10 % (v/v) in warm broth and were rotated in conical flasks at 37°.

Assay of free coagulase. Measured volumes (0·1 or 0·2 ml.) of dilution of culture supernatant in broth were added to 0·2 ml. of a 0·4 % (w/v) solution of bovine fibrinogen containing also 0·5 % (v/v) rabbit plasma in tubes 5·0 × 0·8 cm.; the fibrinogen solution was warmed before the addition of coagulase. Each tube was held against a black background in a warm illuminated box and tapped at intervals until a definite clot appeared; this time was measured accurately. For times up to about 3 min. the reciprocal of the coagulase concentration plotted against the clotting time gave a straight line, the relation between coagulase and clotting time being similar to that found in the case of thrombin + fibrinogen mixtures (Biggs & Macfarlane, 1953). A series of these lines for different preparations of coagulase showed that they intersected the time axis at about 15 sec., which corresponded to the time lag when a large amount of coagulase was used in the test. For periods longer than 3 min. the lines tended to curve, possibly because the end-points were difficult to read accurately. The straight lines extrapolated were then used as an approximate measure of coagulase concentrations for these longer clotting times. A dried preparation of coagulase containing 6000 minimal clotting doses (M.C.D.)/mg. was used as a standard.

Coagulase activator. This was measured in a similar way to free coagulase: 0·1 ml. of coagulase 3000 M.C.D./ml. was added to a mixture containing 0·1 ml. activator solution to be tested and 0·2 ml. 0·4 % (w/v) bovine fibrinogen (Armour's fraction 1). By using a known activator solution as reference, the clotting times plotted against the reciprocal of the activator dilution again gave a straight line.

Bacterial counts. Fully-grown shaken cultures were easily counted in red-cell counting chambers since the cells rarely formed groups of more than two or three. During the logarithmic phase counting soon became inaccurate, and was not used when the staphylococcal clumps became large.

EXPERIMENTAL

That the addition of serum to the medium does not always enhance coagulase production is indicated in Table 1. Strains 2, 59 and 22 (corresponding to the A, B and C strains described earlier (Duthie, 1952)) were unaffected, but

Table 1. *Effect of addition of rabbit serum to culture medium on the production of free coagulase by different strains of staphylococci*

	Incubated with 10 % (v/v) serum for		Incubated without serum for	
	1 hr.	2 hr.	1 hr.	2 hr.
	Coagulase concentration (M.C.D./ml.)			
Strain				
2	2500	3800	3000	3800
59	2000	2450	2100	2600
22	2100	6000	2000	4300
176	4300	5000	550	550
346	2000	1960	270	340

strains 176 and 346 were more typical, in that the addition of serum greatly increased the final yield. A comparison (Table 2) of the sera of a number of animals showed enhancement of production in the case of all those examined. Apart from mouse plasma, which had a smaller enhancing effect, there was no difference between the plasmas and sera of the other animals. Armour's bovine albumin (fraction V) was equal if not superior to any of the sera tested.

Table 2. *Effect of addition of serum* (10 % *v/v*) *of different animals to culture medium on production of coagulase by staphylococcal strain* 346

	Period of incubation of culture	
	1 hr.	2 hr.
	Coagulase concentration (M.C.D./ml.)	
Origin of serum		
Mouse	1320	1320
Guinea-pig	2000	2000
Rabbit	2000	3000
Sheep	1740	1740
Cow	2000	2000
Horse	1800	1800
Pig	3000	3000
Man	2400	2400
Bovine albumin 0·5 % (w/v)	3500	2600
Nil	270	270

Serum fractionation

The results obtained with different fractions of plasma or serum after treatment with ammonium sulphate (Table 3) show that the enhancing effect of plasma or serum on coagulase production was due to the albumin fraction: this was confirmed with the plasmas of several species. Table 4 shows that the addition of increasing amounts of purified bovine albumin (Armour's fraction V) to the medium was accompanied by an increase in the amount of coagulase formed. Since this source of albumin was readily accessible, sterile, and uniform in its results, it was used in subsequent work at a concentration of 0·3 % (w/v) albumin.

Table 3. *Effect of different fractions of human plasma* (*ammonium sulphate precipitation*) *added to the culture medium on the production of coagulase by staphylococcus strain* 346

Final concentration of each fraction equivalent to that present when 10 % (v/v) plasma was added.

	Period of incubation	
	1 hr.	2 hr.
	Coagulase concentration (M.C.D./ml.)	
Material added		
Human plasma 10 % (v/v)	1740	3500
Dialysed human plasma 10 % (v/v)	2400	2400
Human globulin	500	500
Human albumin	3500	2400
Bovine albumin 0·3 % (w/v)	2400	3500

Table 4. *Effect of adding different concentrations of bovine albumin (fraction V) to the culture medium on the formation of coagulase by staphylococcus strain* 346

Coagulase concentration was measured after incubation at 37° for 2 hr.

Albumin (%, w/v)	Coagulase concentration (M.C.D./ml.)
1·0	3000
0·5	2600
0·3	1600
0·15	1540
0·08	1400
0·04	1260

Heat stability and nature of the albumin fraction

When held at 60° for 20 min. the albumin fraction was most stable at pH 2·0 or less. There was an apparent loss at pH 4·6, but this was eliminated when the somewhat cloudy albumin was brought to pH 2 and then rapidly made neutral. There was a marked loss above pH 8·5 which was not reversible.

When 3% (w/v) bovine albumin in 0·1N-HCl was held for 20 min. at temperatures above 60°, there was no loss at 70°, a 50% loss at 90°, and a 75% loss at 100° in the amount of coagulase subsequently formed. Davies (1951) showed that the coagulase enhancing factor in serum was destroyed by pepsin. Table 5 shows that it was also readily damaged by small amounts of crystalline trypsin (flask 2) though not when soya-bean inhibitor was present in the original incubation mixture (flask 3). The later addition of the inhibitor to flask 4 was a safeguard against possible destruction of the coagulase by trypsin, but as will be seen from Table 5, was not necessary in this case. Gelatin, egg albumin, protamine and casein were inactive; egg yolk was less effective than most sera.

Table 5. *Destruction by crystalline trypsin of the factor in bovine albumin which promotes coagulase production*

One volume of each of the following additional mixtures was added to a flask containing 4 vol. of seeded broth. The constituents of the mixtures were in the following concentrations: albumin 1·5% (w/v), trypsin 10^{-5} (w/v), soya-bean inhibitor 10^{-5} (w/v).

		Period of incubation	
		1 hr.	2 hr.
		Coagulase concentration (M.C.D./ml.)	
Flask	Mixture		
1	Albumin, 2 hr., 37°, pH 8	2000	2400
2	Albumin + trypsin, 2 hr., 37°, pH 8	940	880
3	Albumin + soya-bean inhibitor + trypsin, 2 hr., 37°, pH 8	2350	3000
4	Albumin + trypsin, 2 hr., 37°, pH 8, then soya-bean inhibitor added	600	570

Effect of varying the time of albumin addition and inoculum size

Although Davies (1951) stated that coagulase is produced during the latter part of the lag phase, this was not confirmed by his tables nor by the values shown in Table 6. In this experiment four flasks containing a broth + albumin medium were inoculated with falling numbers of staphylococci while a fifth heavily seeded flask contained no albumin. Practically all the coagulase of the most heavily seeded flask containing albumin was formed by the first division. With smaller inocula coagulase production was continuous through the logarithmic phase and in the most lightly seeded flask was equal at the fifth hour (not shown) to that in any of the first three flasks.

Table 6. *Effect of inoculum size on the production of coagulase by staphylococcus strain* 346 *in medium containing* 0·3 % (*w*/*v*) *albumin*

Incubation (hr.)	Flask no.	Count (10^6/ml.)	Coagulase production (M.C.D./ml.)
0	1	166·4	80
	2	41·6	20
	3	10·4	5
	4	2·7	—
	5*	166·4	—
1	1	302	2700
	2	55	790
	3	10	135
	4	2·7	50
	5*	302	550
2	1	640	3700
	2	120	2700
	3	19	420
	4	3·0	50
	5*	640	900
3	1	1200	3700
	2	444	3700
	3	—	1600
	4	5·0	110
	5*	1200	900
4	1	3000	3700
	2	614	3700
	3	—	3000
	4	10	1240
	5*	3000	1240

* No albumin.

For albumin to exert its optimal effect it was essential that it should be added early in the growth cycle; its effect decreased with the number of divisions which had occurred before it was added (Table 7). Thus additions made after 20 min. to the heavily inoculated flasks or after 2 hr. to the more lightly inoculated ones, became progressively less effective with increase of time and hence of cell multiplication. As before, coagulase production was not confined to the lag phase.

Table 7. *Effect of adding bovine albumin* 0·5 % (*w*/*v*) *to cultures of staphylococcus strain* 346 *at different times after seeding*

Bovine albumin added at (min.) after inoculation	Cells (10^6/ml.)	Period of incubation 1 hr.	Period of incubation 3 hr.
		Coagulase formed (M.C.D./ml.)	
10	157·5	1230	4000
20	157	1170	4000
40	237	900	3750
60	430	30	3350
90	—	30	1800
Not added	—	30	—

Bovine albumin added at (hr.) after inoculation	Cells (10^6/ml.)	Period of incubation 3 hr.	Period of incubation 4 hr.
		Coagulase formed (M.C.D./ml.)	
0	11·9	1760	5000
1	12·3	900	3750
2	17·9	700	3800
3	84·0	275	3000
4	—	275	750
Not added	—	275	750

DISCUSSION

Davies (1951), who first studied in detail the action of serum on the production of free coagulase, considered two possibilities: (*a*) that a pro-coagulase is formed in large amounts on the cell surface during the lag phase and is subsequently shed into the medium, in small amounts without serum and in large amounts in its presence; (*b*) that the cells form a pro-coagulase during the lag phase, serum accelerates this formation and when cell division starts pro-coagulase is no longer formed. Davies applied the term 'pro-coagulase' to the material liberated into broth and which, in his terminology, combines with coagulase activator to give coagulase. His 'pro-coagulase' is thus identical with the coagulase of all other authors and his coagulase with the so-called 'coagulase thrombin' of Miale (1949). It is therefore unnecessary to introduce other and more confusing terms.

The present studies, while confirming the importance of serum or, as it now appears, of albumin in the formation of coagulase, do not support some of the conclusions drawn by Davies, nor are the facts covered by the possibilities he put forward. The free coagulase which Davies studied is formed early in the growth cycle and is liberated continuously both in the lag and logarithmic phases. Since no evidence could be obtained in the present studies for the liberation of measurable amounts of free coagulase by lysed or crushed cells grown without albumin, it seems unlikely that the role of albumin is that of a liberator of preformed coagulase. The evidence points to albumin as being directly concerned in coagulase formation or as exercising a protective action on coagulase by preventing its destruction by proteolytic enzymes or surface denaturation. There is at present no evidence in favour of this latter possibility. Lominski, Smith & Morrison (1953) showed that a coagulase-destroying enzyme (possibly a protease) occurred in cultures of weakly coagulase-positive variants or in coagulase-negative strains of staphylococci. Such an enzyme, if present during coagulase production, might cause considerable loss and its inhibition by albumin would explain the enhanced production in the presence of this substance. Unfortunately for this explanation the coagulase-destroying enzyme, like most if not all bacterial proteases (Duthie & Lorenz, 1949) was inhibited by serum globulin and by egg albumin (neither of which affect coagulase production) while serum albumin failed to inhibit it (Lominski, personal communication). Experiments made with purified coagulase in broth showed that while it was readily denatured by shaking in the presence of air, no protection was given by albumin. For these reasons it is thought that the function of albumin is not a protective one.

Strains giving good yields of coagulase in the absence of albumin are rare, and it has so far been impossible to obtain partially purified coagulase type D in a form suitable for the production of antisera. The addition of cadmium sulphate to coagulase grown in an albumin containing medium, gave a precipitate which contained large amounts of albumin. No satisfactory methods for separating the albumin from the coagulase could be discovered and further purification was abandoned.

I wish to thank Mr H. Proom of the Wellcome Research Laboratories and Mr J. C. Francis for many of the staphylococcal strains used. I am very grateful to Dr H. J. Rogers for very helpful discussions and to Miss S. Isherwood and Miss J. Rowlands for valuable technical help. The work was carried out under a research grant from the South-West Metropolitan Regional Hospital Board.

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(*Received* 13 *November* 1953)

MUSHIN, R. & KERR, V. J. (1954). *J. gen. Microbiol.* **10**, 445–451.

Clotting of Citrated Plasma and Citrate Utilization by Intestinal Gram-negative Bacilli

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SUMMARY: Salmonella, shigella, alkalescens-dispar, proteus, paracolon and coliform strains were tested for the presence of a coagulase enzyme as manifested by the ability to clot plasma. The shigella and proteus strains examined gave negative results; a high percentage of the remaining strains clotted citrated plasma but this reaction was due to metabolism of citrate and subsequent liberation of calcium ions and not to a coagulase enzyme. Results depended on the bacterial strain and on the kind and dilution of plasma used. Utilization of citrate in citrated plasma was compared with the ability of the tested strains to attack citrate in Koser's ammonium citrate medium and in sodium citrate peptone medium. The *Shigella* and *Proteus* species examined failed to utilize citrate in any of the media under the conditions of the tests, *Salmonella* species gave fairly uniform results in the three media, while paracolon, coliform and alkalescens-dispar strains differed in their activity, the highest percentage of positive reactions being obtained in plasma-containing media.

In the course of a study of Gram-negative bacilli these organisms were examined to see whether they possessed coagulases capable of clotting plasma. A survey of literature showed that numerous bacterial species have been examined for plasma-coagulating ability. Conflicting results have been obtained, due possibly to different experimental conditions. In the present communication only intestinal Gram-negative bacilli will be considered.

Loeb (1903) used aseptically drawn goose-blood, which was centrifuged and diluted 1/10. He noticed the strong coagulating power of *Staphylococcus aureus* and that *Salmonella typhi* had no apparent effect, while *Pseudomonas aeruginosa* and *Escherichia coli* had some coagulating ability. Much (1908), in experiments with human plasma and citrate, fluoride or hirudin as anticoagulants, reported negative results with *Sal. typhi*, *E. coli*, Friedländer's bacillus and *Proteus* spp. Kemkes (1928) published negative findings in tests with oxalated plasma and the following organisms: *Ps. aeruginosa*, *Ps. fluorescens*, *Sal. typhi*, *Shigella flexneri* and *Sh. dysenteriae*. Lusena (1929) noted that some *Sal. typhi* cultures coagulated decalcified plasma. Gross (1931), found various bacteria, *Sal. typhi*, *Sal. paratyphi*, *Ps. aeruginosa*, shigella and proteus strains, negative in their action on rabbit plasma. Fisher (1936) investigated the coagulating properties of staphylococci on oxalated and citrated plasma and observed that contaminants such as some strains of *Bacillus subtilis* and *Ps. aeruginosa* caused clotting of rabbit but not human plasma, while Friedländer's bacillus, *Alcaligenes* sp., *E. coli* and *Aerobacter aerogenes* did not clot either. Reimer (1936) used various kinds of plasma without addition of anticoagulant and compared the clotting time of plasma mixed with bacterial cultures with that of control plasma. He found no analogy between activity on human and other animal plasmas; *E. coli* accelerated clotting of human plasma, while *Sal. typhi* and *Sal. paratyphi-B* inhibited clotting. Fredericq (1942) made experiments with oxalated plasma and recorded that *Ps. aeruginosa*, *Ps. fluorescens* and *B. subtilis* had a coagulating effect, that of *Ps. aeruginosa* being the strongest.

Rita (1945*a*, *b*) recorded clotting of citrated plasma by *Sal. typhi* and other species of *Salmonella* but found no action on plasma to which oxalate or fluoride had been added. This writer concluded that strains which utilized citrate released calcium ion and thus caused plasma clotting. Rita (1945*c*), in tube experiments, noticed also the slow (8–10 hr.) clotting caused by salmonellas as compared with the quick (1–3 hr.) reaction caused by staphylococci.

Harper & Conway (1948) investigated 8 strains of coliform and 5 of paracolon bacilli and found that 11 of the 13 strains clotted human citrated plasma at 37° within 18 hr.; the clotting reaction was proved to be due to the breakdown of citrate with liberation of calcium ion and not to a coagulase. However, Krech (1952) demonstrated an active agent in filtrates of *E. coli*. He used citrated plasma of man, horse, cow and guinea-pig and found that horse plasma was most actively coagulated and that a temperature of *c*. 16° was the important factor in the test.

The present communication describes the investigation of a collection of Gram-negative intestinal bacilli, including salmonella, shigella, alkalescens-dispar, proteus, coliform and paracolon strains, with regard to clotting action on citrated and heparinized plasma and citrate utilization.

MATERIALS AND METHODS

Organisms. The following strains were examined: 52 salmonellas, 34 shigellas (7 *Sh. dysenteriae*, 18 *Sh. flexneri*, 7 *Sh. boydii*, 2 *Sh. sonnei*), 4 alkalescens-dispar group, 53 proteus (23 *Pr. mirabilis*, 25 *Pr. morganii*, 5 *Pr. vulgaris*), 140 paracolons (91 *Paracolobactrum coliforme* type I, 9 *P. coliforme* type II, 12 *P. intermedium* type I, 5 *P. intermedium* type II, 10 *P. aerogenoides* type I, 11 irregular citrate-negative and 2 irregular citrate-positive) and 14 coliforms (*E. coli* type I). Salmonella strains originated from the National Collection of Type Cultures, shigella and alkalescens-dispar strains were sent by Dr Ewing, U.S.A., while proteus, paracolon and coliform strains were from our own collection isolated from normal and abnormal faecal specimens and classified according to *Bergey's Manual of Determinative Bacteriology* (1948).

Methods. Rabbit and human plasmas were tested. Rabbit blood was collected aseptically by cardiac puncture and mixed in the proportion 9 ml. blood with 1 ml. of 10% (w/v) sodium citrate (final concentration 1%). Human plasma was obtained from a blood bank and contained 4 vol. blood to 1 vol. of 2% (w/v) sodium citrate (final concentration 0·4%). In tests with heparinized plasma 4 drops of the anticoagulant were added to 20 ml. citrated human plasma. The citrate media used included Koser's (1924) ammonium citrate solution and citrate in Bacto-peptone basal medium (Kauffmann, 1951).

Slide coagulase tests were performed with undiluted citrated rabbit plasma. For tube coagulation experiments rabbit plasma was diluted 1/10 or 1/5 with normal saline; the human plasma was diluted 1/4 or 1/2 or used undiluted. In tube tests 0·5 ml. of appropriate plasma was added to 0·5 ml. of an 18 hr. culture of the required organism in Hartley's tryptic broth. The results were recorded after 24 hr. incubation at 37°; negative tubes were kept under observation for 3 days.

Each batch of plasma was tested for clotting capacity by adding 1 drop of 5% calcium chloride to 0·5 ml. plasma. Control slide and tube coagulase tests were included in which known coagulase-positive and coagulase-negative strains of staphylococci were used.

Utilization of citrate in Koser's medium at 37° was considered to be positive when growth was evident after a third successive subculture by inoculation with a straight wire at 48 hr. intervals. Utilization of citrate in the citrate peptone medium was assessed with lead acetate indicator after incubation of the cultures in triplicate sets at 37° for 2, 7 and 14 days.

RESULTS

Clotting of citrated plasma. The slide coagulase tests were uniformly negative. Occasionally granulation slowly took place which at first glance could have been mistaken for clotting, but after some practice it became evident that late and doubtful reactions were non-specific and should be recorded as negative.

Table 1. *Clotting reactions with citrated and heparinized plasma caused by Gram-negative organisms*

Strains	No. of strains tested	Citrated plasma										Heparinized human plasma	
		Rabbit*				Human†							
		Plasma dilution											
		1/10		1/5		1/4		1/2		Undiluted		1/2	
		Citrate (%)											
		0·1		0·2		0·1		0·2		0·4		0·2	
		+	−	+	−	+	−	+	−	+	−	+	−
Salmonella	52	45	7	46	6	7	45	46	6	46	6	0	52
Shigella	34	0	34	0	34	0	34	0	34	0	34	0	34
Alkalescens-dispar	4	4	0	4	0	3	1	4	0	4	0	0	4
Proteus	53	0	53	0	53	0	53	0	53	0	53	0	53
Paracolon	140	92	48	101	39	2	138	107	33	108	32	0	140
Coliform	14	14	0	14	0	0	14	14	0	14	0	0	14

* Diluted 1/10 in 10% (w/v) sodium citrate.
† Diluted 1/5 in 2% (w/v) sodium citrate.
+ = clot; − = no clot.

Table 1 shows that none of the strains investigated clotted heparinized plasma, thus showing the absence of coagulase under the conditions of the test. This was confirmed by tests with oxalated rabbit plasma; in no instance was a positive reaction obtained.

All shigella and proteus strains were uniform in their inability to clot citrated plasma. All the *E. coli* and alkalescens-dispar strains and the majority of salmonella and paracolon strains clotted citrated plasma within 24 hr. With citrated human plasma an increase in the number of positive readings was obtained with 1/2 dilution of plasma (citrate concentration 0·2 %) as compared with 1/4 dilution, but with undiluted plasma only one paracolon strain gave an additional positive reaction. Citrated rabbit plasma was clotted by most strains at 1/10 dilution (citrate concentration 0·1 %) and only a few more strains clotted plasma at 1/5 dilution. With a few exceptions strains clotted both citrated human and rabbit plasma; under the conditions of test 1 salmonella and 10 paracolon strains gave positive readings with human plasma only, while 1 salmonella and 3 paracolon strains acted only on rabbit plasma. Thus the ability to clot citrated plasma depended on the bacterial strain and also on the dilution and kind of plasma used, the concentration of citrate being the important factor. Harper & Conway (1948) showed that clotting takes place when the concentration of citrate is less than that necessary for binding free calcium ions. The clot produced in our tests was soft, occasionally with a slight precipitate and unlike the fibrin-thread type of staphylococcal coagulum which retracts from the walls of the tube.

Additional tests were performed to find out whether a change of pH value was responsible for plasma clotting. Control plasma + sterile broth had a pH of 7·2. Clotted plasma cultures were shaken to break up the clot and the pH measured by means of a glass-electrode. Various cultures showed an increase of pH value to 7·9–8·4; no clotting occurred in control media when the pH was adjusted to 8·4.

Of 140 paracolon strains tested, 16 liquefied gelatin; of these 9 did so within 1–2 days, but the remaining 7 strains acted more slowly. Eight active gelatin-liquefying strains did not clot plasma. Tests with these strains were repeated and the tubes examined every hour, but clot formation followed by solution was not observed.

Table 2. *Utilization of citrate in various media*

				Citrate media			
		Clotting of plasma		Koser		Citrate peptone medium	
		Positive reactions					
Strains	No. of strains tested	No.	%	No.	%	No.	%
Salmonella	52	47	90	46	88	46	88
Shigella	34	0	0	0	0	0	0
Alkalescens-dispar	4	4	100	1	25	1	25
Proteus	53	0	0	0	0	0	0
Paracolon	140	111	80	31	22	42	30
Coliform	14	14	100	0	0	1	7

Citrate utilization. Table 2 shows the results of experiments on citrate utilization in various media: human and rabbit citrated plasma, Koser's ammonium citrate medium and citrate peptone medium. All the *Shigella* and three of the *Proteus* species (*Pr. rettgeri* was not tested) gave negative results in all media. Some proteus strains (*Pr. mirabilis* and *Pr. vulgaris*) showed growth in Koser's medium on first subculture, but growth was not maintained on the second transfer. Readings for the salmonellas ran parallel in all three media, with two exceptions: of 52 strains tested, 45 utilized citrate in all three media and 5 strains (*Sal. paratyphi-A* var. *durazzo*, *Sal. abortus-equi*, *Sal. sendai* and 2 strains of *Sal. typhi*), gave negative results; 1 strain of *Sal. typhi* attacked citrate in rabbit plasma only; *Sal. carrau* utilized citrate in all media except rabbit plasma. Paracolon, coliform and alkalescens-dispar strains gave a much higher percentage of positive reactions with citrated plasma than with Koser's or citrate peptone media. Only 2 paracolon strains utilized citrate in Koser's or citrate peptone media without being able to do so in plasma. Of the 29 paracolon strains which did not clot plasma, 20 were *Paracolobactrum coliforme* type I, one was *P. coliforme* type II, one was *P. aerogenoides* type I, one was *P. intermedium* type II, and 6 were irregular citrate-negative types.

To assess the clotting time of rabbit plasma (diluted 1/10), tests were carried out with a number of paracolon and coliform strains (Table 3). Only one paracolon strain produced a soft clot after 2 hr. incubation, the majority of paracolon and coliform strains gave positive reactions after 10 hr.; no additional positive results were recorded after 21 hr. In contrast to the slow action of the majority of paracolon and coliform strains, our control coagulase-positive staphylococci coagulated plasma in tube tests after 2 hr.

Table 3. *The effect of incubation time on clotting reactions*

Rabbit plasma (diluted 1/10) used to observe clotting

		Time of reading (hr.)							
	No. of strains	1	2	3	4	10	21	24	48
Strains	tested	Positive clotting reactions							
Paracolon	78	0	1	8	14	51	66	66	66
Coliform	13	0	0	0	0	10	13	13	13

DISCUSSION

False positive coagulase reactions with citrated plasma by some salmonella strains (Rita, 1945*a*, *b*) and by some paracolon and coliform strains (Harper & Conway, 1948) were shown to be due to utilization of citrate with liberation of calcium ion. Evans, Buettner & Niven (1952) observed that a similar reaction accounted for the clotting of plasma by certain streptococci.

Our experiments with Gram-negative intestinal bacilli show that the majority of salmonella, paracolon, coliform and alkalescens-dispar strains which clotted human or rabbit citrated plasma did so because of citrate breakdown. On the other hand, all *Shigella* species and three *Proteus* species did not cause clotting. Lominski, Conway, Harper & Rennie (1947) pointed out that some *E. coli* strains which failed to attack citrate in Koser's medium (ammonia as source of nitrogen) attacked citrate in the presence of a different source of nitrogen, e.g. peptone. The *Shigella* and *Proteus* species examined here gave negative results in the three citrate media; this may be helpful in diagnostic work, e.g. when it is difficult to classify an organism as shigella or paracolon, a positive citrate test would exclude shigella. *Pr. mirabilis* and *Pr. vulgaris* strains were reported (Rustigan & Stuart, 1945; Cook, 1948; *Bergey's Manual*, 1948) to utilize citrate, but Kauffmann (1951) listed these species as citrate-negative. It is possible that proteus strains of faecal or non-faecal origin may differ in citrate utilization; our cultures were of faecal origin. It is clear from our findings that attention should be given to the conditions and details of these tests; in our experiments with Koser's medium a positive reading was recorded when growth appeared after three successive subcultures from inoculation with a straight needle at 48 hr. intervals. Rustigan & Stuart (1945) observed that proteus strains grew at different rates on citrate medium. Cook (1948) mentioned that he inoculated Koser's citrate media with a straight wire. The published results, therefore, emphasize the need to standardize citrate utilization tests.

Rita (1945*c*) noticed that *Sal. typhi* strains varied in ability to clot plasma, and suggested that the division of *Sal. typhi* into strains which clotted or did not clot citrated plasma might be useful in epidemiological investigations. Corda (1947) disagreed with this basis for differentiation because of the instability of the character. Our experiments also showed the unreliability of this criterion since results occasionally varied with the batch of plasma used. Krech (1952) described some *E. coli* strains which possessed an active agent in bacteria-free filtrates which was capable of coagulating plasma at room temperature but not at 37°. This, apparently, is a different reaction from that investigated in our study. The ability of *Ps. aeruginosa*, *Ps. fluorescens*, *B. subtilis*, *Chromobacterium prodigiosum* and actinomycetes to clot plasma is attributed by Fredericq (1942, 1946*a*, *b*) to a different factor, namely a secretion of coagulase at 37°. Our work supports the suggestion of Harper & Conway (1948) and of Evans *et al.* (1952) that, to avoid false positive coagulase reactions, tests should be performed with plasmas which contain anticoagulants other than citrate, e.g. oxalate or heparin.

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(*Received* 23 *November* 1953)

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Wavelengths of Bacterial Flagella

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SUMMARY: In sunlight dark-ground microscopy the flagella of a packet of motile sarcina appear as a smooth tail. The tails often stiffen into helices of two different wavelengths, one being twice as long as the other. The short and the long wavelengths are identical in *Sarcina ureae* and *S. agilis*. This phenomenon of two wavelengths, one twice as long as the other, was also found, though less frequently, in certain other bacteria. Otherwise the wavelengths of the flagella of these bacteria appeared specific.

It has been claimed that bacterial flagella are not homologous (Pijper, 1949*a*, *b*; 1951; Pijper, Crocker, van der Walt & Savage, 1953) but fall into different natural groups. It is therefore significant that the novel flagellar feature described here apparently is the exclusive property of one of these groups.

MATERIALS AND METHODS

We are indebted to Dr J. P. van der Walt, now of the South African Council for Scientific and Industrial Research, for isolating *Sarcina ureae* from local garden soil, and to Prof. Annelyse Winkler of Göttingen and Dr E. G. Pringsheim of the Botany School, Cambridge, for a strain of *S. agilis*, and of *Caryophanon latum*, respectively. An H901 culture of *Salmonella typhi* we owe to Dr A. Felix. For flagellar wavelengths of other bacteria we used photomicrographs published by others.

The two *Sarcina* and *Caryophanon* spp. were examined as agar cultures, the flagella being slightly thickened by precipitated agar without having their wavelengths affected (Pijper, 1949*b*). Similarly, *Sal. typhi* was suspended in 0·5% (w/v) methylcellulose in saline.

The sunlight dark-ground microscope technique used was described by Pijper (1946, 1949*b*). Flagellar measurements on the sarcina were made from dark-ground photomicrographs produced by a ×60 Zeiss objective, and a Kontax camera, resulting in a magnification of ×300. The negatives, through a photographic enlarger, were projected at a magnification of ×3000 and the flagellar shapes traced with pencil on paper. The tracings were measured with a pair of compasses and a millimeter scale. Pl. 1, figs. 2–7, and Pl. 2, figs. 8–12, illustrate some of the flagella measured. Assuming that measurements were accurate to 0·3 mm., actual wavelengths (distance from crest to crest) could be expressed with an accuracy of 0·1 μ.

RESULTS

Wavelengths of flagella of sarcina

Packets of *S. ureae* in sunlight dark-ground microscopy showed their flagella (word here used without prejudice) as a faint smooth tail (Pl. 1, fig. 1), very much like the fuzzy-looking tail previously described for *Sal. typhi* (Pijper, 1946, 1949*b*). These tails changed their shape in various ways, but the most common change was a sudden stiffening into a helix (Pl. 1, figs. 2, 3). This transition did not directly affect the motility of the packet. Its causation remains obscure. It might happen to one of several packets in a field, or to most packets in a field. Older cultures showed it more often. After several days of growth most packets had these helical flagella, or the helices had broken off and were floating by themselves. Helices whether loose or still attached tended to combine. Lengthwise 'linking' is shown in Pl. 1, figs. 4–6. The regularity of the coils facilitated transverse 'packing', bringing about the more solid structures of Pl. 1, figs. 5 and 7. Entanglement of 'linked' and 'packed' helices caused pseudo-agglutination (Pl. 1, fig. 5). 'Packing', whilst increasing the width of the helices to a slight degree, did not affect wavelengths.

Gradually the impression was gained that *S. ureae* produced helices of two different wavelengths, and that the one wavelength was double the other. This is clear in Pl. 1, fig. 6, where examples of the two wavelengths are shown in one field, and in Pl. 1, fig. 7, where flagella of two wavelengths are accidentally linked up.

An investigation of *S. agilis* showed similar conditions. Faint fuzzy tails stiffened into helices of short wavelength (Pl. 2, fig. 8) or long wavelength (Pl. 2, fig. 9). Lengthwise linking of short-wavelength helices is shown in Pl. 2, fig. 10, and accidental linking of short and long wavelength flagella in Pl. 2, fig. 11. In Pl. 2, fig. 12, examples of the two wavelengths are seen in the same field. The fundamental wavelengths in *S. ureae* and *S. agilis* appeared the same. *S. ureae* habitually produced more helices of long wavelength than of short wavelength, and *S. agilis* did the opposite.

A large number of measurements seemed needed to decide what the relations really were. This was done as described above, the results are given in Table 1.

Table 1 allows the conclusion that the tails of both *S. ureae* and *S. agilis* change into helices of two kinds, and that in both organisms the one kind of helix has a wavelength of $1{\cdot}6\,\mu$, and the other a wavelength of $3{\cdot}2\,\mu$, thus confirming the impression that one wavelength is double the other.

Observations on other bacteria

The occurrence of helical flagella of two wavelengths, one double the other, was looked for in other bacteria. Our collection of dark-ground photomicrographs of *Sal. typhi* showed several instances, of which Pl. 2, figs. 13 and 14, are examples. The two wavelengths are about $1{\cdot}1$ and $2{\cdot}2\,\mu$. In *Caryophanon latum* straight fuzzy tails can also stiffen into helical structures; Pl. 2, fig. 15, shows two helical flagella of different wavelength attached to one cell, and one wavelength is double the other.

Table 1. *Statistical analysis of measured wavelengths of flagella of* S. ureae *and* S. agilis

	S. ureae		*S. agilis*	
	Short	Long	Short	Long
Mean wavelength (μ.)	1·639	3·193	1·638	3·188
Standard error	0·0054	0·0048	0·0038	0·0068
No. of measurements	141	615	304	298
Confidence limits for mean at:				
5 %	1·639 ± ·011	3·193 ± ·009	1·638 ± ·007	3·188 ± ·013
1 %	1·639 ± ·014	3·193 ± ·012	1·638 ± ·010	3·188 ± ·018

Ratio between short and long wavelengths

	Confidence limits (%)	Minimum	Maximum
S. ureae	5	1·930	1·967
	1	1·924	1·972
S. agilis	5	1·930	1·963
	1	1·923	1·969

Transitions from smooth tails into helical flagella were described for *Proteus vulgaris* (Pijper, 1946, 1949*b*) and for *Bacillus cereus* (Pijper, 1949*b*). At that time no helices of different wavelengths were noticed or photographed. Pietschmann (1942) published a dark-ground photomicrograph of *B. cereus* in 1 % (w/v) tragacanth solution in water where in one field two different wavelengths were visible in stiffened helical flagella. The wavelengths were approximately 2·4 and 1·2 μ. Her only comment was that flagella of *B. cereus* which had stopped moving could show narrow or wide windings. She also showed similar photomicrographs of two bundles of flagella of *Pr. vulgaris*, one having a wavelength double the other, without comment. Neumann (1928) published, also without comment, two photomicrographs of bundles of helical flagella of *Pr. vulgaris* suspended in 5 % (w/v) gum arabic in water; the wavelength of one was *c.* 0·9 μ, and of the other *c.* 1·9 μ.

DISCUSSION

The hard horny excrescences called flagella in spirilla have nothing in common with the soft and fuzzy-looking structures called flagella in sarcina, *Sal. typhi*, *Pr. vulgaris*, *B. cereus*, *C. latum* and allied bacteria. In keeping with this, the flagella of *Spirillum* spp. do not occur with two different wavelengths; their very structure would seem to exclude this (Pijper, 1949*a*, *b*, 1951; Pijper *et al.* 1953). This constitutes a further argument why bacterial flagella cannot be regarded as homologous. Bacterial flagella are generally supposed to have a wavy, spiral (or better, helical) appearance. Flagellar wavelength has not been studied much, probably because the traditional dry preparations were regarded as misleading. Leifson (1951), however, although he only dealt with dried and stained preparations, suggested that perhaps wavelengths might be more important than length. Leifson & Hugh (1954) since found 0·62 μ the average wavelength of twenty-five fixed and stained flagella of a pseudomonad. On this unusually short wavelength they wished to found a new species. Our findings suggest a need for measurements on wet preparations and on larger numbers. Reichert (1909) from dark-ground observations in colloid solutions concluded that in *Sal. typhi* the pitch of the screw-like flagella was 2·5 μ, and of *Pr. vulgaris* 2 μ. In two motile sarcinas he estimated the pitch in dead individuals to be sometimes 2 or 1·8 μ., but usually 3 μ. Weibull (1949) precipitated shaken-off flagella of *Pr. vulgaris* and *B. subtilis* with ammonium sulphate and described the wet flagella as having a period of 2 μ. in *Pr. vulgaris* and 2·5 μ. in *B. subtilis*. Later (1950) he measured the precipitated flagella of ten strains of *Proteus* spp. and confirmed his value of 2 μ. From this he concluded that a definite spiral period is a characteristic feature of bacterial flagella. Neither author had noticed the occurrence, to which we here call attention, of the two wavelengths, one twice the other. Their figures therefore require correction. With this important reservation we confirm that flagellar wavelength is a characteristic feature. Its practical value for classification, however, is further limited by the necessity to perform many measurements on wet preparations, and by the fact that the range of available wavelengths is very limited. Leifson's (1951) observation of a strain of *Sal. wichita* which dissociated into two variants, one showing flagella twice the wavelength of the other, has no bearing on our phenomenon. The two kinds of wavelength were not seen by him in one and the same culture but only in two separate cultures, and moreover in fixed and stained preparations which easily give rise to artefacts. Also, our strain of *Sal. typhi* H 901 can hardly contain two variants. The central coiled filament of the flagellum of *B. brevis* indicated in the electron photographs of de Robertis & Franchi (1951) and the helical fine structure of the flagella of a motile diphtheroid appearing in the electron photographs of Starr & Williams (1952) appear to be of rather too different an order to cause the phenomenon we have described here. Our phenomenon may find its explanation in the molecular structure of this particular kind of flagellum.

Our thanks are due to the South African Council for Scientific and Industrial Research for providing the services of a research assistant and the use of laboratory equipment.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Fast moving small packet of *S. ureae*, with tail; × 1000.

Fig. 2. Small packet of *S. ureae*, tail stiffened into helix of long wavelength; × 1500.

Fig. 3. Small packet of *S. ureae*, tail stiffened into helix of short wavelength; × 1500.

Fig. 4. Several helices of short wavelength of *S. ureae* linked up lengthwise; × 1500.

Fig. 5. Transverse ‘packing’ and lengthwise ‘linking’ of helices of long wavelength of *S. ureae*; × 1500.

Fig. 6. Helical flagella of both long and short wavelength of *S. ureae* in same field, several helices linked up lengthwise; × 1500.

Fig. 7. ‘Packed’ helix of short wavelength linked up accidentally with helix of long wavelength, both of *S. ureae*; × 1500.

Plate 2

Fig. 8. Small packet of *S. agilis*, tail stiffened into helix of short wavelength; × 1500.

Fig. 9. Large packet of *S. agilis*, tail stiffened into helix of long wavelength; × 1500.

Fig. 10. Long stretch of helices of short wavelength of *S. agilis* linked up lengthwise; × 1500.

Fig. 11. Helices of long and short wavelength of *S. agilis*, accidentally linked up; × 1500.

Fig. 12. Helices of long and short wavelength of *S. agilis* in one field; × 1500.

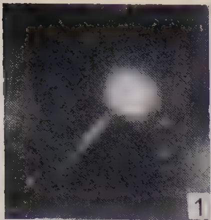
Fig. 13. *Sal. typhi* with helix of short wavelength; × 1500.

Fig. 14. *Sal. typhi* with numerous helices of long wavelength; × 1500.

Fig. 15. *Caryophanon latum* with one helix of short wavelength and one helix of long wavelength; about × 600.

(*Received* 30 *November* 1953)

Journal of General Microbiology, Vol. 10, *No.* 3



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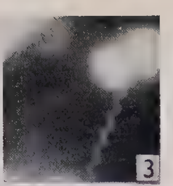

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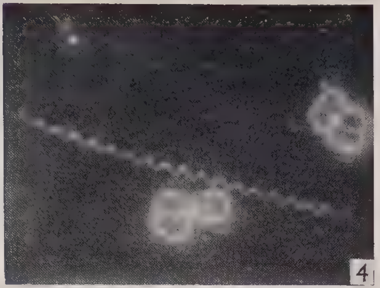

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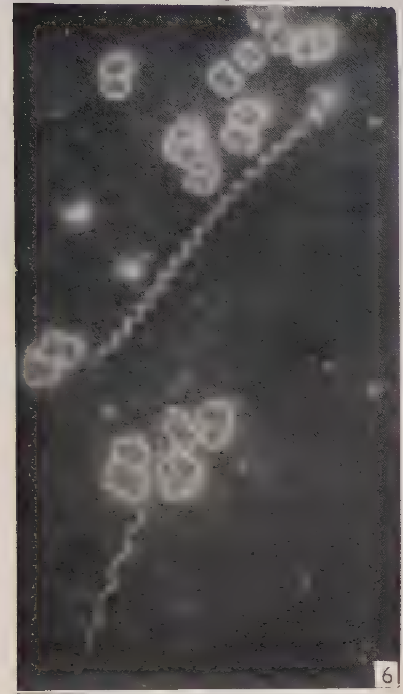

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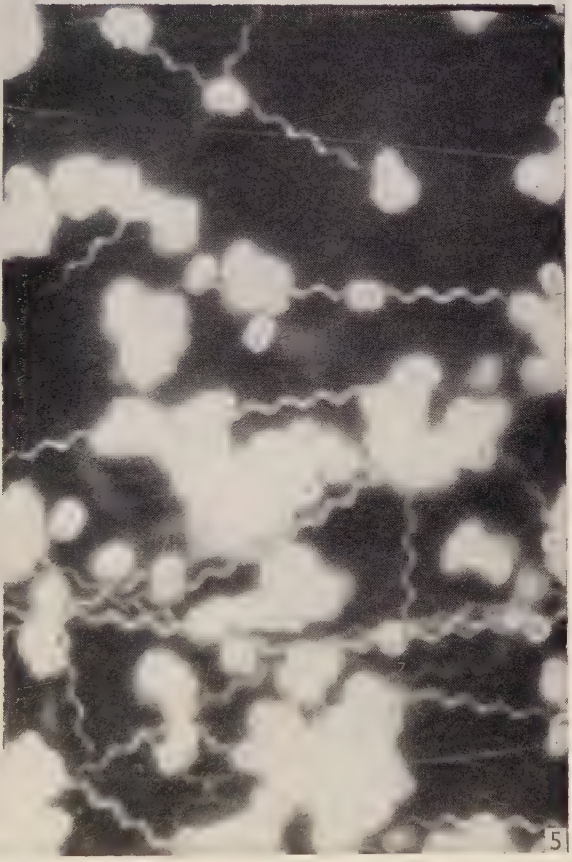

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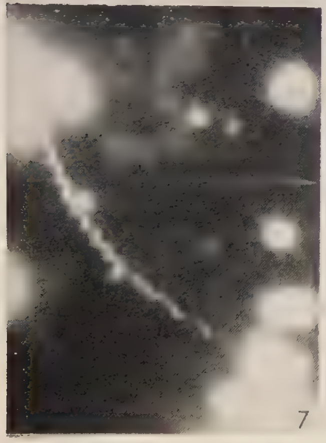

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A. Pijper & G. Abraham—Wavelengths of bacterial flagella. Plate 1

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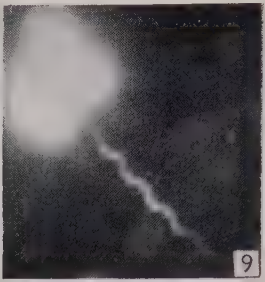
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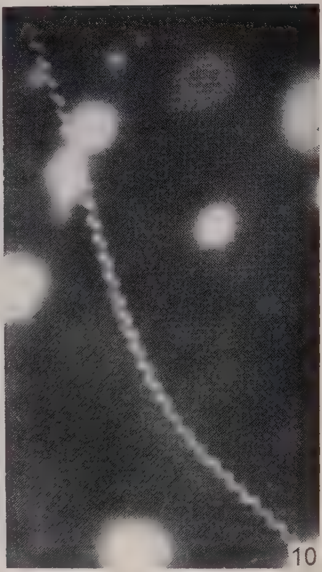
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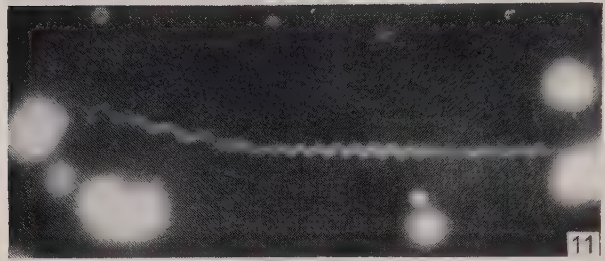
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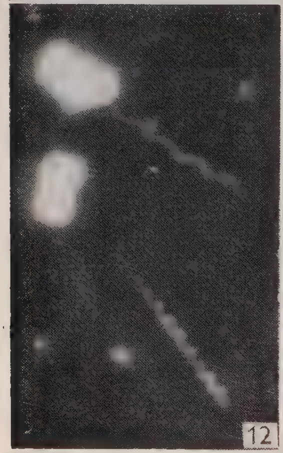
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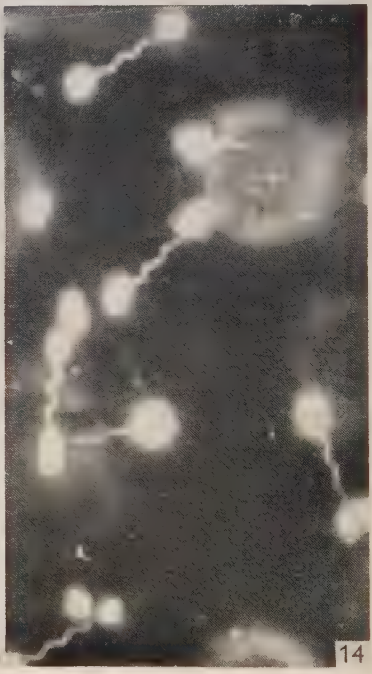
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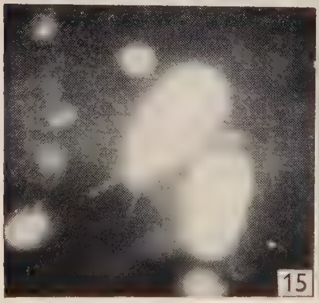
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A. PIJPER & G. ABRAHAM—WAVELENGTHS OF BACTERIAL FLAGELLA. PLATE 2

Donald, H. B. & Isaacs, A. (1954). *J. gen. Microbiol.* **10**, 457–464.

Counts of Influenza Virus Particles

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SUMMARY: Particle counts of influenza virus preparations were made by two independent techniques; there was good agreement in the counts found by the two methods. Counts made on four strains of influenza virus and one strain of 'incomplete' virus showed that at the agglutination end-point there was about one virus particle per red cell. Parallel infectivity measurements of these strains made under optimal conditions showed that about ten virus particles corresponded to one ID 50.

Many studies of the quantitative aspects of influenza virus growth have involved assumptions about the number of virus particles which constitute one infective dose. It has seemed worth while to test these assumptions by counting the number of virus particles seen by electron microscopy which correspond to one egg infective dose. Two independent techniques were used for making the particle counts, the first based on the spray technique used by Luria, Williams & Backus (1951) in which counts are made of the virus particles mixed with polystyrene latex particles of known concentration, and the second based on the method of Dawson & Elford (1949) in which the virus particles are adsorbed on red cell ghosts. In the spray technique the virus particles were counted before and after absorption with red blood cells and the difference gives the number of particles which should be specifically adsorbed to the red cell ghosts. This figure was found to correspond extremely well with the estimated number of virus particles on the surface of the red cell ghosts. In this way an estimate of the number of virus particles in different preparations of influenza virus was obtained, and this figure correlated with simultaneous measurements of the agglutination titre and infectivity of the preparation. This paper describes the results obtained.

Materials

Virus strains: Influenza A—PR8 (1934), Melbourne (MEL) (1935) and A/England/1/51 strains; Influenza B—Lee (1940). The virus seeds were stored in capillary tubes at −65°.

Latex preparations. Polystyrene latex suspensions containing $1{\cdot}55 \times 10^{13}$ particles/ml. were kindly supplied by Dr R. C. Backus. The particle concentration was checked from the weight of a dried sample of suspension and the mass of a single latex sphere as computed from measurements of density and diameter. Dilutions of 1/500 of the standard suspension were found most suitable.

Bovine serum albumin. Armour Laboratories Crystallized Bovine Plasma Albumin.

Phosphate buffered saline. 0·9% NaCl, M/33 phosphate, pH 6·7.

Saponin. One ml. 6% (w/v) saponin was used to lake 75 ml. of a 1% (v/v) suspension of red cells.

Sodium metaperiodate. Crystalline chemically pure $NaIO_4$ (2·14 g.) dissolved in 1000 ml. of phosphate buffered saline to give a stock solution of 0·01 M. A dilution of 0·001 M was used to treat red cells.

Glycerol saline. Glycerol 1% (v/v) in phosphate buffered saline.

Broth saline. Bacteriological (Hedley-Wright) broth diluted with an equal volume of normal saline.

Red cells. Fowls were bled from the wing vein into sodium citrate solution, the red cells washed thrice in normal saline, stored at 4° and used within 3 days. For agglutination titrations the cells were made up in a 0·5% (v/v) suspension in normal saline calibrated in a photoelectric densitometer.

Methods

Preparation of virus for testing. Precautions were taken to ensure that the virus used in these experiments was of high infectivity and was tested for infectivity under optimal conditions. Fertile eggs (10-day) were inoculated intra-allantoically with 0·05 ml. of a high dilution (von Magnus, 1946), usually 10^{-5}, of seed virus. The eggs were incubated at 35° and were harvested before the virus had reached its maximal titre in order to minimize deterioration in infectivity which might occur on prolonged incubation. Immediately after harvesting the infectivity and agglutination titres of the virus were measured, and some material was stored in capillary tubes at −65° for further tests at a later date. The remaining material was centrifuged lightly to remove tissue debris, dialysed overnight against distilled water and then used for spraying. Agglutination tests were carried out after centrifugation and dialysis so that any alteration in the concentration of virus as a result of these procedures could be observed. Usually there was no change in titre after centrifugation and dialysis, but sometimes a slight decrease in titre was found. In some experiments a large decrease in titre occurred after dialysis and such preparations were discarded. 'Incomplete' virus was prepared as described later.

Haemagglutination titration. Titrations were carried out in plastic plates and the results read by the pattern method. Serial twofold dilutions of virus (0·25 ml.) were made in saline, and an equal volume of 0·5% (v/v) chick red cells added. The cells were allowed to settle and the agglutination end-point taken as the tube showing partial (i.e. slight but clearly detectable) agglutination. Where the partial agglutination end-point was not visible it was obtained by interpolation. Since many workers have used 1% red cell suspensions, the titres obtained were adjusted so that they express the number of agglutinating doses per ml. of virus for 0·25 ml. of 1% red cells.

Infectivity titration. In the preliminary titration serial tenfold dilutions of virus were prepared in chilled broth saline and 0·05 ml. amounts of 10^{-7}, 10^{-8}, 10^{-9} dilutions were inoculated by the allantoic route into groups of five 10- to 12-day eggs. In a second titration with virus stored at −65°, 3·16-fold dilutions were chosen around the end-point and five groups of four eggs were used. The eggs were incubated for 72 hr. at 35° and then tested for agglutinins. The EID50 was calculated by the method of Reed & Muench (1938) and the average of the two titrations was used to calculate the number of EID50/ml. of original material. Agreement between the two titrations was fairly good; on the average the second titration gave a value of $0{\cdot}17 \pm 0{\cdot}45$ log units lower than the first.

Virus particle assay by spraying technique. A gun similar to that of Luria, *et al.* (1951) was used. It consisted of two concentric glass tubes, the inner tube being drawn out to a fine bore with uniform end cross-section of approximately 0·15 mm., and the outer tube having an inlet for air under pressure. A pressure of 20 lb./sq.in. was used and, to diminish turbulence, the specimen grids were mounted on a thin upright support at a distance of 11 in. from the tip of the gun. The preparation to be examined was mixed with a measured sample of the polystyrene latex suspension to give a final concentration of $3{\cdot}1 \times 10^{10}$ latex particles/ml. Before spraying a few crystals of purified bovine serum albumin were added to give a concentration of about 0·001% (w/v) and this helped to mark the edges of the droplet patterns clearly. Preparations were mixed thoroughly by pipetting, sprayed immediately on to collodion-coated grids and shadowed with gold-manganin before being examined in the electron microscope. The gun was washed thoroughly with distilled water between the examination of various strains.

Counts were made of the number of influenza virus particles in individual drops, several hundred particles of each type being counted in each experiment. To test whether a particular ratio of influenza virus to latex particles was significantly constant throughout an experiment, the correlation coefficient (r) between the counts of particles of the two types in individual droplets was calculated. A high and significant correlation was found in all the experiments quoted (i.e. $r > 0{\cdot}6$; $P < 0{\cdot}01$).

To test the specificity of the influenza virus particles counted, a measured sample of the infected allantoic fluid was absorbed with red blood cells as in the next technique. The supernatant from this was dialysed overnight against distilled water and then mixed with polystyrene latex and sprayed as above. Usually this absorption removed the great majority of virus particles from the allantoic fluid.

Virus particle assay by red cell absorption technique. Fowl red blood cells were haemolysed with saponin and washed thoroughly with phosphate buffered saline. Influenza-infected allantoic fluid was spun lightly to remove any blood cells or tissue debris and then mixed with the red cell ghosts. The ghosts were treated before contact with the virus with 0·001 M-sodium metaperiodate solution (Fazekas de St Groth, 1948) a treatment which modifies the receptors on the surface of the cell membrane in such a way that virus absorption is unimpaired while elution is almost completely prevented. Absorption and all subsequent washings were carried out at 0°. After an hour's contact with the infected allantoic fluid the cells were centrifuged at 2500 rev./min. for 5 min. and the supernatant fluid removed. The cells were then resuspended in phosphate buffered saline fixed in 0·1% osmic acid for

30 min., washed with distilled water and mounted on electron microscope grids coated with collodion. The final distilled water washings were carried out at low speed (500 rev./min.) and the ghosts mounted carefully from very dilute suspensions so that flatly mounted cells relatively free from wrinkles and swelling of the nucleus were obtained. Specimens were examined in the electron microscope by direct transmission and after shadowing with gold-manganin. Counts were made of the average number of virus particles per red cell ghost assuming a random distribution of particles over the membrane. This assumption was justified by counts made of the numbers of particles present in ruled areas of the ghost surface; the distribution of particles in these areas did not differ significantly from that expected on the Poisson distribution (see Pl. 2, fig. 4). Only those particles which cast a shadow were counted and identified as being on the top surface of the membrane. Areas to which particle counts referred were measured directly from the electron micrographs, by using transparent graph paper. In order to obtain an estimate of the total number of particles/red cell, the figure obtained was increased to allow for the total countable area, then increased by 15% to allow for the area masked by the nucleus, and finally doubed for the reverse side of the ghost. The total number of ghosts used in a particular preparation was counted in a haemocytometer cell and thus a figure was obtained for the number of virus particles in one ml. of the original allantoic fluid.

Electron microscopy. A Siemens Electron Microscope was used with 70μ. objective aperture and an accelerating voltage of 50 kV. A magnification of 5000 on the screen was used for most micrographs. Some of the later micrographs were taken with a new Siemens machine using a 50μ. objective aperture.

RESULTS

The results of electron microscopy are illustrated in Pl. 1, figs. 1, 2 and Pl. 2, figs. 3, 4. Pl. 1, fig. 1, shows a microdrop containing influenza virus and latex particles, and a corner of this microdrop is shown at a higher magnification in Pl. 1, fig. 2. The latex particles are about 250 mμ. in diameter and extremely regular in size and shape. The smaller virus particles, of about 100 mμ. diameter, could be counted easily after a little practice. They were of characteristic appearance and were removed from the microdrops by absorption with red cells; Pl. 2, fig. 3, shows the appearance after absorption with red cells. Pl. 2, fig. 4, shows the virus particles now adsorbed on the surface of the red cell ghost. In a few preparations, occasional clumps of about five to six particles were seen in sprayed material, but never in virus adsorbed on the red cell surface; the particles in such clumps were counted individually. Occasional filaments were seen in preparations of MEL and incomplete PR8 virus, and they were common with A/England/1/51 virus; the filaments were counted singly.

Table 1 shows the complete results of a single experiment with the strain MEL. In order to assess the results of an experiment a number of ratios were calculated: the ratio of particles counted/agglutinating dose by the two counting techniques used; the ratio of infective doses/agglutinating dose when infectivity was measured at the beginning of the experiment under optimal conditions; the ratio of virus particles/infective dose, obtained by dividing the first ratio by the second. Estimates of the ratio of particles counted/agglutinating dose by the two techniques showed an extremely good agreement; the spray technique gave a value of 0·07 ± 0·15 log unit higher than the red cell technique. In general, however, we have preferred to put greater weight on the counts obtained by the spraying technique since it involved fewer assumptions.

Table 1. *Counts of particles, egg-infectivity and agglutinating titres of a preparation of MEL virus*

Particle count/ml. by spray technique	$10^{10\cdot7}$
Particle count/ml. by red cell absorption technique	$10^{10\cdot8}$
Haemagglutinin titre/ml. of original virus	$10^{3\cdot7}$
Haemagglutinin titre/ml. of sprayed material	$10^{3\cdot6}$
Haemagglutinin titre/ml. of adsorbed virus	$10^{3\cdot6}$
Infectivity titre/ml. of original virus	$10^{9\cdot9}$

Table 2. *Ratio of particles/agglutinating dose*

Strain	Description	Ratio (log)		
PR8	A1934	7·3	7·4	7·2
MEL	A1935	7·1	7·3	7·1
A/England/1/51	A1951	7·3	7·1	6·9
Lee	B1940	7·3	7·3	7·2
Incomplete PR8	3rd undiluted passage	7·3	7·2	7·2

Table 2 shows the ratio of the number of virus particles counted by the spray technique to the number of agglutinating doses/ml. of suspensions of four strains of influenza virus. The results of three experiments with each strain are presented and in addition preparations of 'incomplete' virus (von Magnus, 1946) of strain PR8 were also examined. The ratios were surprisingly constant and, with two exceptions, varied between $10^{7\cdot1}$ and $10^{7\cdot3}$ with an overall mean of $10^{7\cdot21}$. There was as great a variation between different measurements of a single strain as between different strains of virus. Counts of the number of red cells in 0·25 ml. of a 1% suspension of red cells gave figures of $10^{7\cdot1}$ to $10^{7\cdot2}$; hence we conclude that at the agglutination end-point there was about one virus particle/red cell, with the strains we used.* The lowest ratio found was with a strain of A/England/1/51 virus in which there were numerous filamentous forms and we have other evidence to suggest that this factor may lower the ratio.

Table 3 shows the ratio of infective doses (ID50)/agglutinating dose for the same preparations. The ratios in most experiments do not differ greatly from those found by other workers (e.g. von Magnus, 1951; Fazekas de St Groth & Cairns, 1952), although two preparations of PR8 on repeated titration gave rather lower ratios. When these two preparations are excluded, the mean ratio of ID50 dose/agglutinating dose for the other 'complete' virus strains was

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* Dr R. W. Schlesinger and Dr G. Werner tell us that they have reached a similar conclusion from counts made by the red cell technique.

Table 3. *Ratio of egg infective doses/agglutinating dose*

Strain	Description	Ratio (log)		
PR8	A1934	5·7	6·2	5·3
MEL	A1935	6·2	6·0	6·2
A/England/1/51	A1951	6·2	6·1	6·1
Lee	B1940	6·2	6·2	5·9
Incomplete PR8	3rd undiluted passage	4·2	4·4	4·2

$10^{6 \cdot 13}$. Slight differences in the ratio found by different workers might depend on the particular agglutination end-points used. The 'incomplete' PR8 was obtained by making three passages of undiluted allantoic fluid at 24 hr. intervals (von Magnus, 1951). The ratios obtained with 'incomplete' PR8 have been consistently lower than those found with standard virus, but have never been so low as those obtained by von Magnus (1951). This finding has been made in our laboratory on a number of occasions, and we do not yet know whether the difference from von Magnus's results is due to virus or egg variation, or to other causes.

Table 4. *Ratio of particles/egg infective dose*

Strain	Description	Ratio (log)		
PR8	A1934	1·6	1·2	1·9
MEL	A1935	0·9	1·3	0·9
A/England/1/51	A1951	1·1	1·0	0·8
Lee	B1940	1·1	1·1	1·3
Incomplete PR8	3rd undiluted passage	3·1	2·8	3·0

Table 4 shows the ratio of virus particles/ID50 dose for the same strains. With the standard strains the ratios found were all of the order of 10 particles/ID50 dose, and for 'incomplete' PR8 the figure was about 1000.

DISCUSSION

Measurements of the number of virus particles per infective dose with a strain of bacteriophage by Luria *et al.* (1951) showed that one virus particle corresponded roughly to one infective dose. Previously Friedewald & Pickels (1944) calculated that about ten virus particles corresponded to one infective dose. This figure was reached as a result of estimates of the weight of a virus particle from a knowledge of its particle size and density. However, their conclusion has not been generally accepted (e.g. Henle, 1950). Their technique was similar to that of Smadel, Rivers & Pickels (1939) who found that, on the average, 4·2 vaccinia virus particles corresponded to one infective dose.

Another method of estimating the number of influenza virus particles/infective dose was that of Fazekas de St Groth & Cairns (1952) who measured the percentage of eggs which became infected after inoculation with closely spaced dilutions of virus. Their experimental dosage-response curve was less steep than that of a theoretical curve based on the assumptions that one virus particle was sufficient to initiate infection and that there was no factor of host variation. They interpreted this result to mean that one virus particle might be sufficient to initiate infection but that there was also a factor of host resistance which tended to lower the probability of infection. However, the experimental result might equally be interpreted on the assumption that considerably more than one virus particle was required to initiate infection and that there was a correspondingly greater factor of host resistance. This interpretation has experimental support since Fulton & Armitage (1951) found that the slopes of the dosage-response curves in infectivity titrations of influenza virus by egg and tissue culture techniques did not differ significantly, although the tissue culture technique is roughly 40 times less sensitive than the egg technique. In view of this and of the present findings it may be, therefore, that some of the conclusions reached by Cairns, Fazekas de St Groth & Edney (1952) require re-interpretation.*

One implication of our results concerns ideas about 'incomplete' virus. Such a name now seems inappropriate if ten particles of 'complete' virus are required to initiate infection. At present we have no information on whether preparations of 'complete' and 'incomplete' virus contain fully infective and fully non-infective virus in different proportions. It would seem more appropriate, therefore, in the present state of knowledge, to refer to preparations of virus as having a given probability of initiating infection. Thus, in our experiments standard virus would contain particles with a probability of about 0·1 and 'incomplete' virus, particles with a probability of about 0·001, of initiating infection. Description in these terms does not commit us to any theory of the mechanisms which govern the probability of initiating infection in advance of information.

Another implication of our results concerns the yield of virus from a single infective dose. Henle, Henle & Rosenberg (1947) found that, on the average, a single infective dose yielded about 100 infective doses after one 'cycle' of multiplication; further 'cycles' were prevented by a 'blocking' dose of inactive influenza virus. In view of the present results it would appear that one infective dose, or ten virus particles, yielded about 1000 virus particles in a single cycle. Of the ten infecting particles, however, only one particle on the average would initiate infection. Hence, the 'burst size', estimated as the number of virus particles resulting from infection by one virus particle in one 'cycle' of multiplication would be about 1000 on the average.

The methods which we have used for measuring the infectivity of influenza virus are the most sensitive in general use at present. However, Liu & Henle (1953) have shown that some eggs injected with small doses ($\frac{1}{4}$–$\frac{1}{8}$ ID 50) of influenza virus may show no agglutinin after incubation for 72 hr. but may yet reveal virus on inoculation into further eggs. This finding, in keeping with the present results, focuses attention on the factors which govern the probability of initiating infection. Elucidation of these factors might help greatly the understanding of the processes of influenza virus multiplication.

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* Luria (1953) states: "The numerical relation between successful particles cannot be decided by titration or by any other statistical tests, but only by actual counts or determinations of particle numbers."

We should like to thank Dr Forrest Fulton with whom we have had many valuable discussions on this work. It is a pleasure to acknowledge the technical help given by Mrs I. M. Honey, Mr O. Green and Mr E. Owen.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Microdrop containing polystyrene latex and Lee strain of influenza virus. The larger dense spheres are the latex particles, the small flatter particles indicated by arrows are the elementary bodies of the virus. Gold-manganin shadowed. × 7,500.

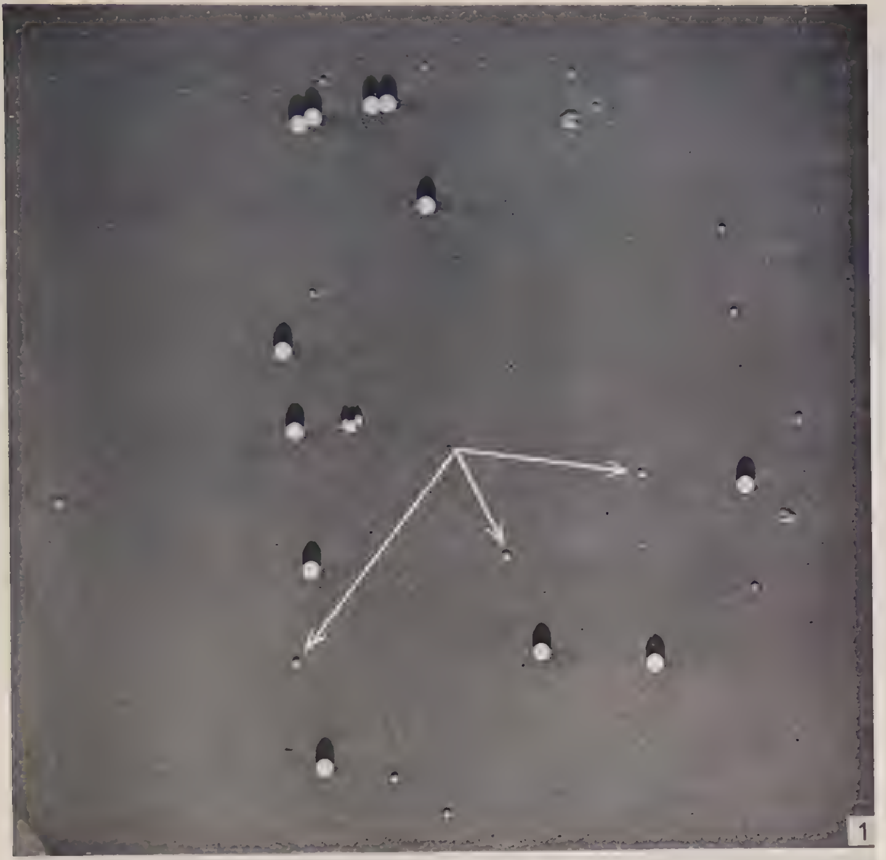
Fig. 2. Portion of a field similar to that of fig. 1 showing two latex and two virus particles. Gold-manganin shadowed. × 30,000.

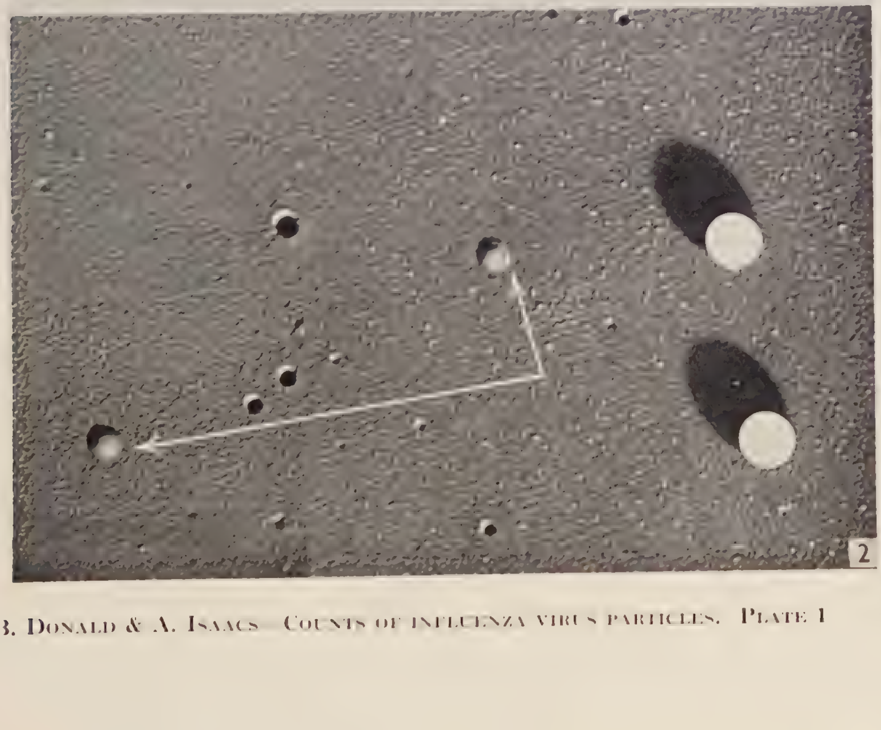
PLATE 2

Fig. 3. Microdrop showing infected allantoic fluid after absorption with red cells. Polystyrene latex particles but no influenza virus particles are present. Gold-manganin shadowed. × 10,000.

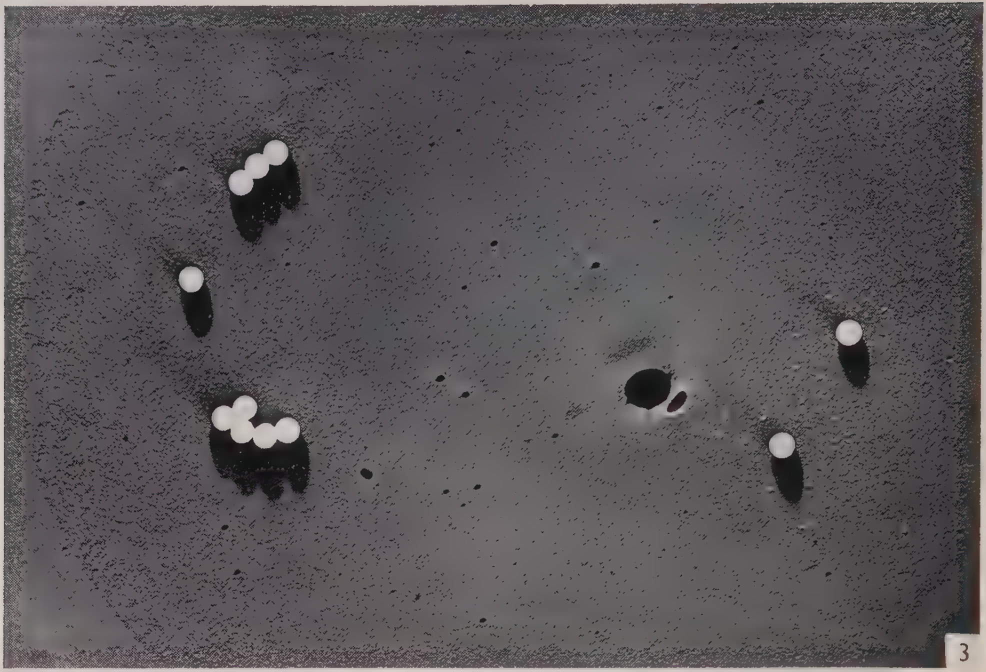
Fig. 4. Particles of Lee strain of influenza virus adsorbed on to a red cell ghost. Gold-manganin shadowed. × 10,000.

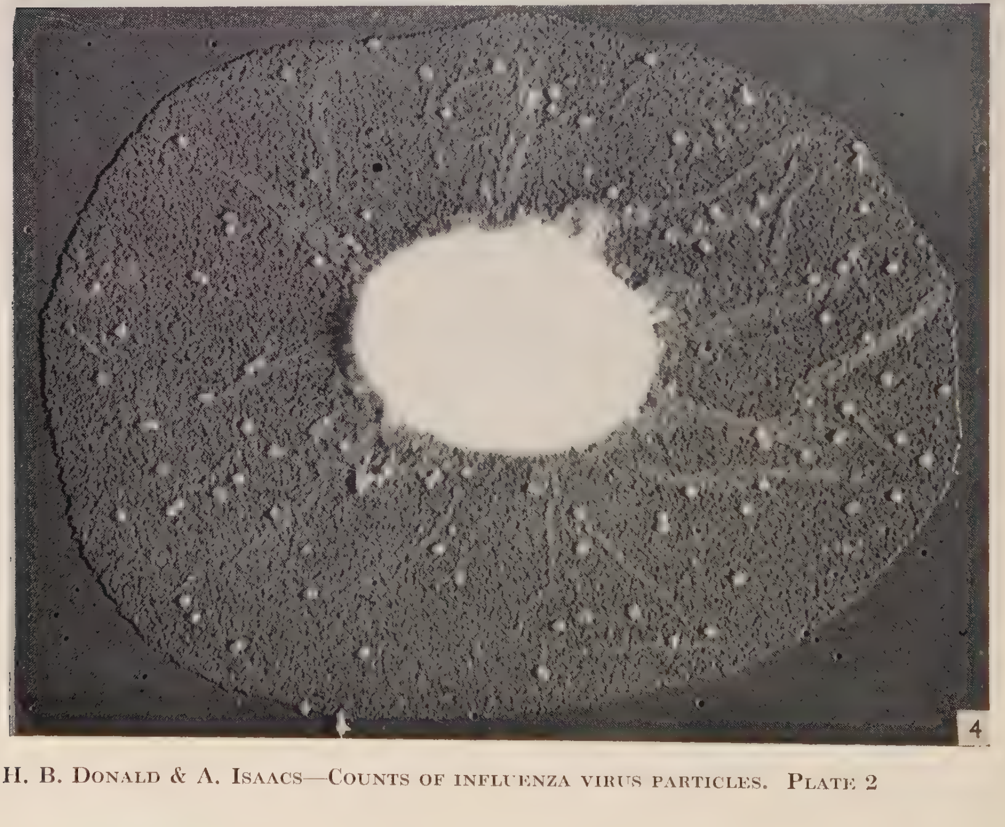
(*Received* 2 *December* 1953)





H. B. DONALD & A. ISAACS—COUNTS OF INFLUENZA VIRUS PARTICLES. PLATE 1





H. B. DONALD & A. ISAACS—COUNTS OF INFLUENZA VIRUS PARTICLES. PLATE 2

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Rules of Nomenclature for Fungi and Bacteria

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SUMMARY: Attention is drawn to certain requirements of the current International Botanical and Bacteriological Codes of Nomenclature, with special reference to the denoting of categories, the proposal of new species, and the changing of names; three procedures commonly encountered by authors and editors. The requirements for the proposal of new specific names of fungi and bacteria are:

General requirements. The name chosen must be in Latin binomial form and should be coined according to the practices set out in the Codes (Bact. Code, Rules, 6, 27, 28, Appendix A; Bot. Code, Art. 33, 82, Rec. 83A).

The name must be effectively published. (It is desirable that scientific journals in which new taxa are proposed should indicate the exact date of publication of the parts.)

The name must be validated by a concise description (the diagnosis) of the diagnostic features of the new taxon. (The diagnostic features should not have to be deduced from an exhaustive account of the organism possibly spread over several pages.)

The description should, whenever possible, be accompanied by illustrations of which the scale and the specimen(s) on which they are based should be stated.

All measurements should be in the metric system.

The date of collection or isolation of the type material should be given together with the precise locality and the substrate or host (the last being designated by its scientific name).

The location of the type should be stated.

The etymology of the name should be explained.

Special requirements for fungi. The diagnosis must be in Latin (Bot. Code, Art. 44).

The type specimen should be deposited, if possible, in a national herbarium, and if the type is a collection and portions are deposited in several herbaria then one of these isotypes should be designated as the holotype.

Special requirements for bacteria. No Latin diagnosis is required but 'in works written in a language unfamiliar to the majority of workers in bacteriology, it is recommended that the authors publish simultaneously the diagnosis in a more familiar language' (Bact. Code, Rec. 12a).

Subcultures of the type strains should be deposited at one or more of the national culture collections.

Micro-organisms belong to several kingdoms, and workers in microbiology have to be familiar with the codes for international nomenclature agreed upon by zoologists, botanists and bacteriologists. There is now general agreement among biologists on the fundamental bases for scientific nomenclature, and the three codes, though independent, show many features in common and differ from one another only in minor and often rather trivial technical details. In a previous article (Ainsworth, 1948) some of the similarities and differences between the zoological and botanical codes were indicated, and though the general principles are still unchanged a number of the statements made in that review need modification because a new Botanical Code was adopted by the 1950 International Botanical Congress and the Bacteriological Code, approved by the fourth International Congress for Microbiology (1947), has been modified by decisions of the fifth and sixth International Congresses for Microbiology held in Rio de Janeiro (1950) and in Rome (1953), respectively. The purpose of the present article is, therefore, to draw attention to certain requirements of the current Botanical and Bacteriological Codes with special reference to fungi and bacteria and, while not absolving the systematist from a close and critical study of the original texts, to attempt to increase the awareness of microbiologists about some general aspects of nomenclature and perhaps to increase respect for internationally acceptable names.

The international rules of nomenclature

The *International Rules of Zoological Nomenclature* were instituted in 1901 and have remained substantially unchanged apart from the series of more than 200 'Opinions' on matters of their interpretation given by the International Commission on Zoological Nomenclature. The 1953 International Zoological Congress did, however, sanction the issue of a revised version of the Code (see Hemming, 1953). The *International Rules of Botanical Nomenclature* first appeared in 1906, were re-issued in 1935, and the current version adopted by the Stockholm Congress in 1950, appeared in 1952 under the new title of *International Code of Botanical Nomenclature.* The bacteriologists' Code, which concerns 'bacteria, related organisms, and the viruses', is the most recent. The tentative *International Bacteriological Code of Nomenclature*, as adopted by the 1947 International Microbiological Congress, was published in 1948 and reprinted in this *Journal* in the following year (*J. gen. Microbiol.* 1949, **3**, 444–62). At the 1953 Rome Congress it was agreed that there should be prompt publication of a revised code as the *International Code of Nomenclature of Bacteria and Viruses*, with a short title of *Bacteriological Code.* Unlike botanists, bacteriologists have a permanent Judicial Commission responsible for their Code and for issuing, on request, formal Opinions on the interpretation of the rules and on the status of names. Such Opinions and other proposals for changes or interpretations of the Code are published in the *International Bulletin of Bacteriological Nomenclature and Taxonomy.* Decisions made and published by the International Committee on Bacteriological Nomenclature and by the Judicial Commission are automatically confirmed at the next International Microbiological Congress if they have not been challenged in the meantime. Although botanists have no Judicial Commission there is a series of standing Congress committees which make various recommendations in connexion with the Code; before submission to an International Botanical Congress such recommendations are now normally published in *Taxon*, the official news bulletin of the International Association for Plant Taxonomy.

The Botanical Code is divided into principles, rules, and recommendations which are set out in a series of eighty-three numbered Articles. The Bacteriological Code, which is set out as a series of numbered Principles and Rules, closely resembles the Botanical Code, and unless stated to the contrary, or clearly inapplicable, any rule or recommendation mentioned without comment may be taken to apply to both codes.

The scope of nomenclature

Before considering the requirements of the Codes it is necessary to stress the subservience of nomenclature to taxonomy. Rules of nomenclature are of little help in devising systems of classification, in determining what shall or shall not be included in a particular taxonomic group, or in answering such questions as what constitutes a 'good' species. These are all matters of taxonomy and are therefore questions for individual judgement. This reflexion of taxonomy by nomenclature explains why the frequent appeals by applied biologists for stability in the nomenclature of the relatively few organisms in which they are interested cannot be met when there are still taxonomic uncertainties. Rules of nomenclature ensure uniformity in the form and precision in the application of scientific names after the taxonomic decisions have been taken. Judgement is more rarely involved in their application. The codes are arbitrary sets of rules devised because zoology and bacteriology like botany 'cannot make satisfactory progress without a precise system of nomenclature which is used by the great majority...in all countries' (Bot. Code, Art. 1). A code of nomenclature is not a penal code and there is no compulsion, and so 'should be simple and founded on considerations sufficiently clear and forcible for everyone to comprehend and be disposed to accept' (Bot. Code, Art. 3).

The objectives of nomenclature are '(1) to aim at fixity of names' and '(2) to avoid or to reject the use of forms and names which may cause error or ambiguity or throw science into confusion' (Bot. Code, Art. 4). These aims are in general achieved by the 'law of priority' and by the use of nomenclatural types. The law of priority does not over-ride all other considerations; for example a name published without a description of the organism or a reference to a previously published description, has no standing in nomenclature.

Nomenclature thus deals with the *terms* which denote the categories of taxonomic groups and the relative ranks of these categories and the *names* which are applied to the individual taxonomic groups or *taxa* (sing. *taxon*), a term introduced in the latest Botanical and Bacteriological Codes.

The new Codes also distinguish clearly for the first time between *legitimate* and *illegitimate* names or epithets,* which are names or epithets chosen in accordance with or contrary to the rules, respectively, and the *correct* name of a taxon with a particular circumscription, position, and rank, which is the name that must be adopted under the rules. This last ensures that an organism can have as many *correct* names as there are different views on its taxonomy, but only one correct name for each taxon with a given circumscription.

The Botanical and Bacteriological Codes are intended to give guidance in every eventuality and they are therefore rather complex. In the following sections attempts are made to indicate the principal requirements of the codes for denoting categories, proposing new species, and changing names (or avoiding the changing of names), the procedures most commonly encountered by authors and editors.

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* A specific epithet (the 'trivial name' of zoologists) is the second component of a Latin binomial name of which the generic name is the first.

Terms and categories

'Every individual is treated as belonging to a number of categories of consecutive rank and consecutively subordinate of which species is the basic one.'

This quotation from Principle 7 of the new Bacteriological Code expresses a familiar and current view. Equally familiar are the supra-specific categories (genus, family, order, class, division) and the sub-specific categories (subspecies, variety, form); categories which may be further subdivided to meet the demands of any particular situation.

The supra-generic categories may be distinguished at a glance by characteristic suffixes: family names end in- *aceae* (subfamilies in *-oideae*, tribes in *-eae*), names of orders in *-ales* (suborders in *-ineae*). For plants the suffix for classes is *-opsida* and for divisions *-phyta* except for fungi for which the suffix *-mycota* is recommended for divisions and *-mycetes* for classes, classes of Algae being distinguished by the suffix *-phyceae*.

In general the stem of a family name is a generic name, which, for bacteria, should be that of the type genus (e.g. Spirochaetaceae from *Spirochaeta*), that for an order a family name (e.g. Spirochaetales from Spirochaetaceae), etc.

Under the Botanical Code the relative order of the categories must not be changed and names given to misplaced terms are treated as not validly published (Art. 15). A special exception is, however, made for the names of subdivisions of genera in Fries's *Systema Mycologicum* which are treated as validly published although termed 'tribes', a term correctly denoting subdivisions of a family.

New species

The proposal of a new species implies that the new species is distinct from the often large number of related species already proposed; for fungi, for example, that it is a distinct taxon from the hundred thousand species proposed; the fact that more than half of these have already been relegated to synonymy or rejected suggests a need for great caution and for sound taxonomic work. The Codes are not however concerned with the soundness of the taxonomy but only with the regulation of the new name.

A specific name must be in Latin binomial form and the Codes offer detailed advice on what is good taste in the coining of names. (There is nothing in the Bacteriological Code, however, to prevent the use of common names, and these should be encouraged.) Publication must be both *effective*, that is by the distribution of printed matter (but after 1 January 1953 not as tradesmen's catalogues or newspapers, Bot. Code, Art. 39), and *valid* (effective publication conforming to the special requirements of the Codes). Among the requirements of the Botanical Code for the valid publication is the necessity, on and from 1 January 1935, for names of new taxa of recent plants, the bacteria excepted, to be accompanied by a Latin diagnosis (Art. 44). Many authors follow, or attempt to follow, this ruling but other competent workers ignore this requirement and publish the diagnosis in one or more modern languages. Such names are therefore technically invalid but they would rarely if ever be rejected on the score of merely lacking a Latin diagnosis, and it is possible that a Botanical Congress will eventually decide to allow some relief in this connexion (see the argument advanced by Lund (1953) for the use of modern languages in place of Latin for algal taxa).

The application of the names of taxa is given precision by the use of nomenclatural types but under the new Botanical Code the rules of typification (and priority) do not apply to the names of taxa above the rank of order (Art. 26). The nomenclatural type of an order is a family, that of a family a genus, that of a genus a species, that of a species or sub-specific taxon a type specimen, which may be a herbarium specimen or culture; in certain cases it may be merely a published description with or without illustrations.The nomenclatural type indicates the essential features of any taxon, but its scope is left to the judgement of taxonomists. A taxon may be treated so narrowly as to include only the type, it may be treated so broadly as to cover one or more related taxa; in such instances the name of the taxon must be changed as soon as its circumscription covers a taxon bearing an earlier name which then takes precedence (see below).

For higher plants and animals the nomenclatural type is a single specimen, for fungi it is more frequently a single collection which may comprise more than one individual. Such material should be most carefully preserved and its whereabouts stated after the diagnosis, together with details of the place and date of collection or isolation and, if a parasite, by the scientific name of the host.

'Nomenclatural types of bacterial species obviously cannot be the (air) dried or preserved specimens used for higher plants or animals. A type (specimen) may be either a living culture [*culture* is to be interpreted as including every method of maintaining organisms in a living state (in a medium, in a host by passage, in cells or exudates, or desiccated)] maintained in a bacteriological laboratory, more particularly in one of the national type culture collections, or it may be a description' (Bact. Code, Rec. 9). A *type culture* is defined as 'a living culture of an organism which is a descendant of the original culture or isolation from which the author who first described the organism made his original description, which culture has been maintained pure, and which agrees in its characters with the original description' (Bact. Code, Rec. 9).

Authors of new species of bacteria are urged to deposit cultures in one of the major culture collections. When a type culture no longer exists the Judicial Commission may designate a substitute type culture (neotype) as the type; this was done in designating the Marburg strain as the type culture of *Bacillus subtilis* Cohn, the type species of the genus *Bacillus*.

Name changes

Changes of name may be necessitated for technical reasons connected with the Codes.

The name may not be the earliest one. The official starting-point for bacterial nomenclature is Linnaeus, *Species Plantarum*, Ed. 1, 1 May 1753,* and this is also the starting-point for the nomenclature of higher plants, Lichens, Myxomycetes, and, with certain exceptions, Algae. For Fungi, however, the nomenclature starts from Persoon, *Synopsis Methodica Fungorum*, 31 December 1801 (for Uredinales, Ustilaginales, and Gasteromycetes) and Fries, *Systema Mycologicum*, Vol. I, 1 January 1821 (for other groups). Under the Codes the legitimate name is the first to be validly published after the official starting-point for the nomenclature of the group in question. Names in use before the appropriate starting-point are invalid and take their priority status from the date at which they were taken up by a later author. For example, the maize smut must be known as *Ustilago maydis* (DC.) Corda and not as *Ustilago zeae* (Beckm. ex Schw.) Unger because although the epithet *zeae* was first published in 1768 it was not validated until 1822, seven years after the introduction of the epithet *maydis* by De Candolle for the same fungus.

There may be an earlier *homonym*, that is the identical name may have been used before for another taxon. The generic name *Gnomoniopsis* Stoneman, 1898, was changed to *Glomerella* because of the earlier *Gnomoniopsis* Berlese, 1892. Botanists and zoologists do not consider the same names when applied to taxa in the plant and animal kingdoms to be homonyms—*Drosophila* as applied to a genus of toadstools and *Drosophila* as applied to a genus of fruit flies are both legitimate names. 'Bacteriological nomenclature and botanical nomenclature are interdependent in the sense that a name of a bacterial group is to be rejected if it is a later homonym of the name of any plant group' (Bact. Code, Prin. 3) but bacteriological nomenclature is independent of zoological nomenclature with the exception of protozoology. *Listerella* Pirie, *Pfeifferella* Buchanan, *Phytomonas* Bergey *et al.*, and *Rhizomonas* Orla-Jensen are among the bacterial names that must be rejected because they are later homonyms of protozoan genera (see *Int. Bull. bact. Nom. Tax.* **3**, 109, 1953). To provide a new name for *Listerella* is not quite straightforward as the other name used for this genus, *Listeria* Pirie, 1940, is a later homonym of a plant, *Listeria* Necker, 1790. The plant name is not in use and the Botanical Committee on Bacteria is to be asked to recommend the rejection of *Listeria* Necker, and so release *Listeria* for use by bacteriologists.

Another reason for changes of name under the Botanical and Bacteriological Codes is because the binomial is a *tautonym*, the specific epithet exactly repeats the generic name, e.g. the specific epithet of *Sphaeria nummularia* Fr. when transferred to *Nummularia* was changed to *bulliardi* by Tulasne because *Nummularia nummularia* would have been a tautonym. Zoologists find no difficulty in accepting tautonyms, and their use in botany would often, as in the example given, indicate the type species of the genus.

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* Many bacteriologists think that the starting date for bacterial nomenclature should be much later, and certainly in the era of pure cultures. In 1950 the subcommittee on viruses recommended that the starting date of virus nomenclature should be some time in the future.

For pleomorphic fungi, that is to say for those exhibiting two or more states in the life cycle, the Phycomycetes excepted, the first valid name or epithet applied to the perfect state (the state bearing basidiospores, ascospores, or other sexually produced spores) takes precedence, and so the discovery of the perfect state of a fungus necessitates a change of name, but the reworded Art. 69 (the old Art. 57) makes it clear that the usual practice of keeping in use the names of imperfect states in works referring to such states is acceptable. For example, the discovery that *Diplocarpon rosae* Wolf was the perfect state of the imperfect fungus *Actinonema rosae* (Lib.) Fr. necessitated a change in name of the pathogen, a change that need not be accepted in Britain where the perfect state is unknown.

The most important name-changes result from taxonomic work. One species may be considered to comprise more than one element and so a new specific or subspecific taxon is proposed. When a species or other taxon is divided into two or more taxa the original name must be retained for one of them, i.e. for that element which is based on the type of the original taxon. Also, according to the new Botanical Code (Art. 34), the proposal of a new variety of a species automatically creates a second subordinated taxon of the same rank which has as its nomenclatural type the type of the original taxon. Further, this second taxon, the one based on the nomenclatural type of the next higher taxon, is designated by repeating the epithet of the higher taxon unaltered but without the citation of an author's name, e.g. the proposal of *Scopulariopsis brevicaulis* Bain. var. *alba* Thom automatically created a variety *Scopulariopsis brevicaulis* Bain. var. *brevicaulis* based on the type of the species and the name *Scopulariopsis brevicaulis* would now cover both varieties.

The same requirement exists in bacteriology (Rule 7); if an author subdivided *Staphylococcus pyogenes* into two varieties, *aureus* and *albus*, he would automatically create a third variety *Staphylococcus pyogenes* var. *pyogenes*.

More frequently, taxonomic work may suggest that a species would be better classified in another genus, to which it is then transferred, the name of the author of the original species being inserted in parenthesis before that of the author making the transfer, e.g. *Xanthomonas hyacinthi* (Wakker) Dowson (syn. *Bacterium hyacinthi* Wakker). Under the Botanical Code such transfers are from 1 January 1953, only valid 'when the *basonym* (the name-bringing or epithet-bringing synonym) is clearly indicated with its author and the place and date of publication' (Art. 42).

Finally a name may be changed because taxonomic studies are considered to show that two taxa are the same; when the name of the earlier is applied to the new grouping the later name is cited as a synonym, e.g. *Erwinia carotovora* (Jones) Holland (syn. *Bacillus phytophthorus* Appel).

Attention must be drawn to the two very different types of synonyms illustrated by the last two examples. In the first the synonymy is 'obligate', it is one of definition, in the second it is 'facultative', for it is a matter of the taxonomist's opinion. In citing synonymies it is useful to differentiate these two types of synonyms by three- and two-bar identity signs, e.g. *Bacterium hyacinthi* ≡ *Xanthomonas hyacinthi*; *Bacillus phytophthorus* = *Erwinia carotovora*.

In passing it may be noted that misidentifications should not be included in the synonymy but added after it. A misapplied name should be indicated by the words '*auct. non*' followed by the name of the original author and the bibliographical reference (Bot. Code, Rec. 60F). For example, the misidentification of the rust fungus *Uredo gladioli* Requien as the gladiolus smut fungus by Oudemans could be recorded under *Urocystis gladiolicola*, after any synonyms, as *Uredo gladioli* auct. non Requien, Oudemans, *Enumer. Syst. Fung.* **1**, 1215, 1216, 1919.

Conservation

In order to prevent the undesirable consequences which sometimes follow the rigid application of the rules both the Botanical and Bacteriological Codes provide procedures by which a name can be retained in use as an exception. Such names are said to be 'conserved', a fact that should be indicated on formal occasions by the addition of *nomen conservandum* (nom. conserv.) after the name; a rejected name is indicated by *nomen rejiciendum* (nom. rej.). The most usual reason for the need to invoke Congress to conserve a name is to prevent inconvenience resulting from the re-introduction of a neglected earlier name, e.g. of *Syringospora* Quinq., 1868 for *Candida* Berkhout, 1923; also *Chlorobacterium* Guillebeau, 1890, a generic name never used, was rejected to allow the conservation of *Chlorobacterium* Lauterborn, 1915 (type *Chlorobacterium symbioticum* Lauterborn). Another reason is involved when it is desirable to retain a name in use for a taxon which excludes the original nomenclatural type. The current Botanical Code lists genera of fungi, algae, and other groups which have been conserved.

Generic names of certain bacteria have already been conserved under the Bacteriological Code, e.g. *Bacillus* Cohn (see St John-Brooks & Breed, 1937). The generic name *Bacterium* and the family name Bacteriaceae have been rejected by the Nomenclature Committee and will be the subject of Opinion No. 4 (revised). (For Opinion No. 4 as originally promulgated, see Cowan, 1953). Authors must thus now allocate to other genera those species that they would have included in *Bacterium*. For example, *Bacterium coli* would be correctly named *Escherichia coli*. The taxonomic position of *Bacterium aerogenes* is more difficult, and an author must answer the question 'Can I distinguish between *Klebsiella* and *Aerobacter*?' If he can answer 'Yes' then he can use *Aerobacter*, but if the answer is 'No' *Klebsiella* is the generic name to be used. When a binomial is needed for the water bacteriologist's 'intermediate coliforms', the combination *Escherichia freundii* is correct. Paracolons can often be allocated to the genera *Escherichia* or *Klebsiella* (or *Aerobacter*) and, when necessary, can be called *Escherichia dispar*, *E. alkalescens*, etc. Also, the Gram-positive rods classified in the genus *Bacterium* in the 6th edition of the *Bergey Manual* must be allocated among other genera such as *Kurthia* and the new genus *Brevibacterium*, specially proposed by Breed for the Gram-positive rods that cannot be assigned to a genus.

The family name Bacteriaceae having been rejected, a request has been made to the Nomenclature Committee that the incorrect Enterobacteriaceae might be allowed as an exception to replace the rejected name because other correctly coined names (i.e. formed by adding the suffix *-aceae* to the stem of a generic name) such as Escherichiaceae, Proteaceae, Klebsiaceae, Salmonaceae, Shigaceae, though possible are all unpleasant and difficult to pronounce.

Other bacterial generic names approved for conservation at Rome included *Gallionella* Ehrenberg (type *G. ferruginae*), *Beggiatoa*, *Klebsiella*, *Kurthia*, *Neisseria*, *Nocardia*, *Pasteurella* and *Leptothricia*, and among those rejected were *Chlorobacterium* Guillebeau, *Babesia*, *Bacteriopsis*, *Pseudospira*, *Pseudospirillum* and some other names that have not appeared in recent literature.

So far all International Botanical and Zoological Congresses have refused to accept the principle of the conservation of specific names but it appears not unlikely that botanists may adopt the principle of the rejection of specific names which would achieve the same objective as conservation of specific names without many of the disadvantages (see *Taxon*, **1**, 78, 1952). Bacteriologists are more daring, and at Rome the names *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*, were all declared legitimate and the specific epithets of the last three were conserved against any earlier synonyms. Type strains are to be designated by the Enterobacteriaceae Subcommittee.

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The Effect of the γ- and δ-isomers of Hexachloro*cyclo*hexane on the Growth, Fermentation and Respiration of three species of Yeast

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SUMMARY: The growth of three yeast species was inhibited by δ-hexachloro*cyclo*hexane more strongly than by the γ-isomer; the degree of inhibition was related to the inositol requirement of the given yeast. The fermentation of glucose was inhibited only by the δ-isomer and the effect was not reversed by inositol. In one species oxygen uptake was inhibited more strongly by the δ-isomer than by the γ-isomer; only the inhibition caused by the γ-isomer was affected by inositol.

Since Slade (1945) suggested that the toxic effect of γ-hexachloro*cyclo*hexane ('gammexane') might be due to analogue antagonism between this substance and *meso*-inositol a considerable amount of work has been devoted to studying the effect of the isomeric hexachloro*cyclo*hexanes on the growth of a variety of organisms and tissues. Following the demonstration by van Vloten, Kruissink, Strijk & Bijvoet (1948) that it is the δ-isomer whose configuration corresponds to that of *meso*-inositol (hereafter referred to as inositol) the simple theory of antagonism due to stereochemical resemblance had to be abandoned. Although the evidence is somewhat conflicting it seems that both γ- and δ-isomers possess inhibitory or toxic properties for certain biological systems, and that in some cases the inhibition may be partially suppressed by inositol. Kirkwood & Phillips (1946) reported that inositol overcame the effect of gammexane on a strain of yeast for which inositol was an essential nutrient. Fuller, Barratt & Tatum (1950) studied the effect of the γ-isomer on the growth of *Neurospora crassa*, using the wild type and an inositol-requiring mutant. They demonstrated an inhibition which was not annulled by inositol in both strains, and a second type of inhibition annulled by inositol in the mutant strain only. These experiments were criticized by Schopfer (1951) who reported a variable antagonism between the δ-isomer and inositol, quercitol or mytilitol for the inositol-less Neurospora when grown in liquid medium. Fuller *et al.* (1950) did not use the δ-isomer; the antagonism between this isomer and inositol has received comparatively little attention. The present work records some observations on the effect of the γ- and δ-isomers of hexachloro*cyclo*hexane on certain aspects of the metabolism of three species of yeast which differ in their requirement for inositol.

MATERIALS AND METHODS

The organisms used were: *Kloeckera apiculata* (*brevis*) (exacting for inositol); *Saccharomyces cerevisiae* Hansen 77 (non-exacting); *S. carlsbergensis* 4228 (intermediate). The culture medium was that of Hopkins & Pennington (1947) supplemented where necessary with inositol. For the preparation of cell suspensions cultures were grown for 18 hr. (36 hr. in the case of *S. cerevisiae*) at 30° in the liquid medium after which the cells were centrifuged, washed 6 times with 0·02 M-KH_2PO_4 (pH 5·6) solution and finally suspended in the same solution diluted as required to obtain a suitable suspension. Since the hexachloro*cyclo*hexanes are very sparingly soluble in water (although somewhat more so in the presence of sugars), stock solutions (0·24 g./100 ml.) were made in ethanol and suitable amounts of these solutions added to the aqueous media.

Growth of the organisms. Portions (9 ml.) of basal medium were placed in 25 ml. flasks and suitable quantities of an ethanol solution of the required isomer added; the total volume was made up to 9·6 ml. with water or inositol solution after which 1 ml. of the standard yeast suspension was added. After incubation at 30° for 18 hr. the crop of cells was removed by centrifugation, uniformly suspended in 25 ml. of 0·02 M-phosphate and cell counts carried out by haemocytometer. In each test five replicate experiments were performed.

Carbon dioxide output. CO_2 evolution was measured in the Warburg apparatus at 30°. A suitable yeast suspension in phosphate buffer (1 ml.), together with 0·05 ml. hexachloro*cyclo*hexane solution and 1 ml. water (or inositol solution), was placed in the main compartment of the Warburg flask and 0·6 ml. of 0·1 M-glucose in the side tube. After equilibration the glucose solution was run into the main flask and manometer readings taken at 15 min. intervals during periods up to 3 hr.

Oxygen uptake was measured in the Warburg apparatus under similar conditions, except that carbon dioxide was absorbed by KOH placed in the centre cup.

RESULTS

Growth

The results (Figs. 1–3) indicated that the γ- and δ-isomers were to some extent inhibitory to the growth of all three yeasts; the δ-isomer always gave a more marked effect than the γ-isomer for a given concentration. The maximum inhibition was always produced, by both isomers, when the organisms were growing in a deficiency of inositol; even *S. cerevisiae*, considered to be non-exacting, showed a slight response to inositol during an 18 hr. growth period (though not during 30 hr.); in complete absence of inositol it was strongly inhibited by either isomer. This inhibition was, however, almost entirely abolished by 15 μg. inositol/ml. (Fig. 3). In the presence of an adequate amount of inositol, inhibition by the γ-isomer was greatest for *K. apiculata* and least for *S. cerevisiae*, with *S. carlsbergensis* intermediate; this was in accordance with the respective nutrient requirements of the different organisms for inositol. No such agreement appeared with the δ-isomer for, although *S. cerevisiae* was most strongly inhibited, *S. carlsbergensis* appeared to be more sensitive than *K. apiculata*. Inhibition by the δ-isomer was always less affected by inositol than inhibition produced by the γ-isomer, but in no instance was the inhibition completely overcome by inositol; the degree of annulment of inhibition reached a certain figure and there remained constant in spite of increased inositol concentrations (see Table 1).

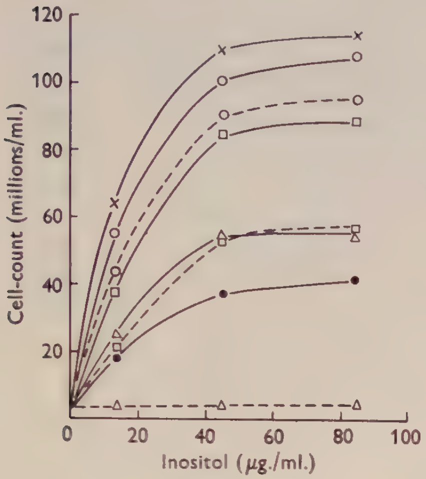

Fig. 1

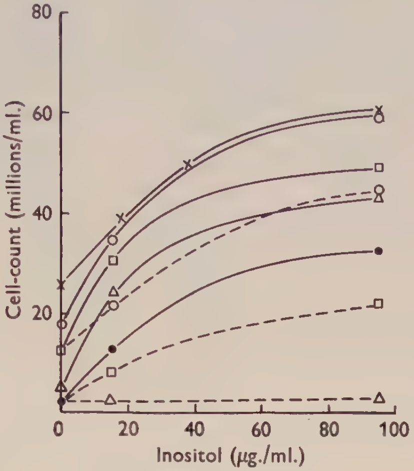

Fig. 2

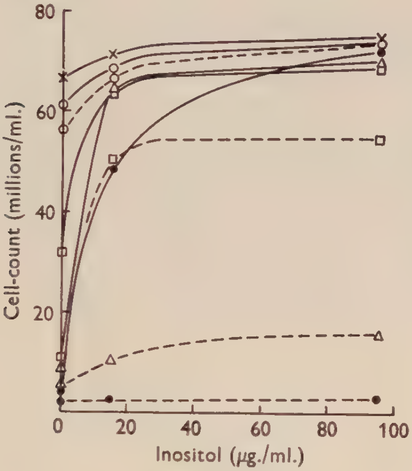

Fig. 3

Figs. 1–3. Growth (cell count after 18 hr.) of *K. apiculata*, *S. carlsbergensis* 4228 and *S. cerevisiae* Hansen 77, respectively, in presence of inositol and isomers of hexachloro*cyclo*hexane. Concentrations of isomer: —×—×—, 4·3 μg./ml.; —○—○—, 10·8 μg./ml.; —□—□—, 21·6 mg./μl.; —△—△—, 43·2 μg./ml.; —●—●—, 86·4 μg./ml. Continuous line, γ-isomer; broken line, δ-isomer.

CO_2 *output*

The γ-isomer had no inhibitory effect on the amount or the rate of CO_2 evolution, and in fact an appreciable increase in the amount evolved in 3 hr. was usually observed (Figs. 4–6). The δ-isomer strongly inhibited fermentation by all three organisms, and inositol (up to 1 mg./ml.) did not decrease the degree of inhibition. No connexion was evident between the inositol requirement (for growth) and sensitivity to the δ-isomer.

Table 1. *The effect of different concentrations of inositol and the γ- and δ-isomers of hexachlorocyclohexane on growth of certain yeasts*

Kloeckera apiculata

	Inositol (μg./ml.)			
	15	30	45	85
γ-isomer (μg./ml.)	Inhibition of growth (% of normal)			
4·3	14	11·5	9	6
10·8	41	30	23	23
21·6	61	54	51	51
43·2	72	68	62·5	63·5
δ-isomer (μg./ml.)				
4·3	31	23	17	17
10·8	67	59	52	51
21·6	(100)	(100)	(100)	(100)

Saccharomyces carlsbergensis 4228

γ-isomer (μg./ml.)	0	15	50	95
4·3	29	13	4	3
10·8	54	22	18	18
21·6	81	37	31	29·5
43·2	(100)	65·5	51	46
δ-isomer (μg./ml.)				
4·3	54	46	33	31
10·8	(100)	79·5	69	65
21·6	(100)	—	—	(100)

Saccharomyces cerevisiae Hansen 77

	Inositol (μg./ml.)		
	0	15	95
γ-isomer (μg./ml.)	Inhibiton of growth (% of normal)		
4·3	9	5·5	4
10·8	51	11	5·5
21·6	81	11	5·5
43·2	93	32	5·5
δ-isomer (μg./ml.)			
4·3	16	8·5	4
10·8	76·5	29	28
21·6	92	86	80
43·2	(100)	(100)	(100)

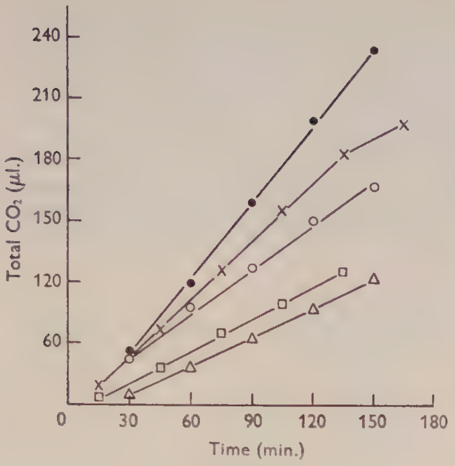

Fig. 4

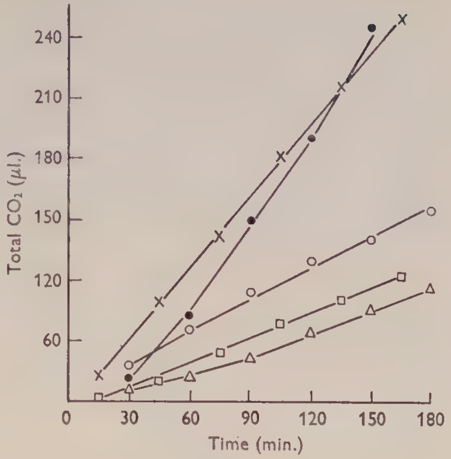

Fig. 5

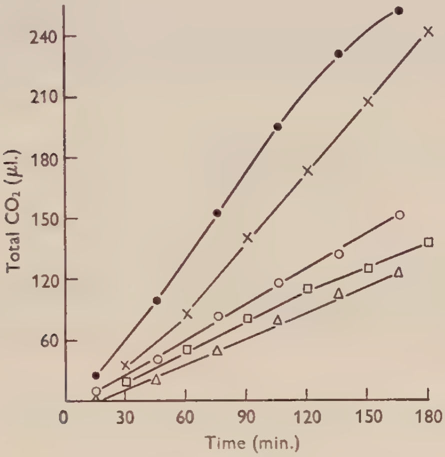

Fig. 6

Figs. 4–6. Carbon dioxide output during 3 hr. by *K. apiculata*, *S. carlsbergensis* 4228 and *S. cerevisiae* Hansen 77, respectively, in presence of hexachloro*cyclo*hexane. Concentrations of isomer: —×—×—, 0 μg./ml. isomer; —●—●—, 45 μg./ml. γ-isomer; —○—○—, 45 μg./ml. δ-isomer; —□—□—, 90 μg./ml. δ-isomer; —△—△—, 135 μg./ml. δ-isomer.

Oxygen uptake

With *S. carlsbergensis* both isomers were inhibitory, the δ-isomer much more than the γ-isomer. A slight decrease of the effect of the γ-isomer was produced by a considerable excess of inositol; under similar conditions no decrease in the inhibition by the δ-isomer was noted (Fig. 7). These experiments were carried out with vigorous young cultures of yeast, and in those experiments concerned with oxygen uptake or CO_2 output the observations were begun not more than 30 min. after the cells were first exposed to the chloro-compounds.

Smith (1951), who investigated oxygen uptake by *S. carlsbergensis*, suggested that the response of the organism to the γ-isomer and to inositol was to some extent dependent on the condition of the yeast culture, i.e. on the age of the culture and the length of time of exposure to the inhibitor. In our hands cultures whose age varied from 15 to 96 hr. showed no differences in response to the γ-isomer as regards growth. The final amount of growth of cultures which had been held at 2° for periods of 1–5 days in the presence of different amounts of the γ-isomer did not differ significantly from controls without the γ-isomer. As regards respiration the only notably different response was that of a culture of *S. carlsbergensis* which had been held at 2° for 5 days in the presence of 100 μg. δ-isomer/ml. The respiration of this culture was completely inhibited but was appreciably restored by inositol (0·4 mg./ml.). However, examination of cells which had been allowed to stand in contact with the δ-isomer revealed a certain amount of disintegration, and differences in behaviour may have been due to this. In general, in all these experiments the δ-isomer produced a more marked inhibition of respiration than the γ-isomer, and respiration was not significantly restored by excess of inositol.

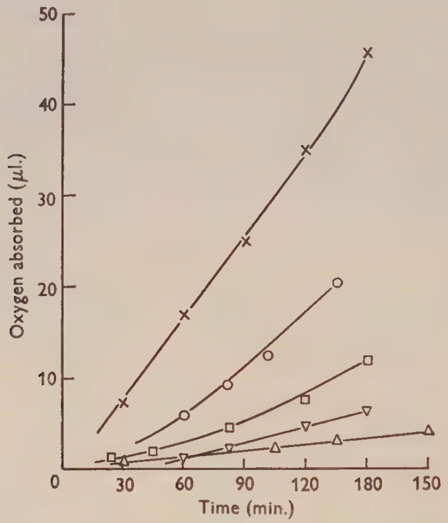

Fig. 7. Oxygen uptake during 3 hr. by *S. carlsbergensis* 4228 in the presence of inositol and isomers of hexachloro*cyclo*hexane. —×—×—, 0 μg./ml. inositol + 0 μg./ml. isomer; —▽—▽—, 13·5 μg./ml. δ-isomer; —△—△—, 135 μg./ml. δ-isomer + 350 μg./ml. inositol; —□—□—, 135 μg./ml. γ-isomer; —○—○—, 135 μg./ml. γ-isomer + 350 μg./ml. inositol.

Note on the solubilities of the isomers of hexachlorocyclohexane. The extremely low solubilities of the isomers in water introduced uncertainty as to the exact concentration of the isomer in solution. In some experiments where the concentration of the γ- or δ-isomer exceeded *c.* 100 μg./ml., although no precipitate appeared when the medium was prepared some precipitation took place later, largely masked, however, by the yeast cells present. It seemed that the upper limits of true solution *in the medium* were *c.* 75 and 100 μg./ml.

for the γ- and δ-isomers, respectively. Nevertheless, when 'concentrations' supposedly greater were used there was a further response by the organisms. This may have been due to adsorption of excess of the chloro-compound at the surface of the cells. In any event the quantitative relation between the concentration of chloro-compounds and the response produced cannot be regarded as established until methods for determining true solubility can be applied and it is therefore not possible accurately to compare the inhibitory power of the two isomers except at very low concentrations.

DISCUSSION

It thus appeared that several aspects of the metabolic activities of all three yeasts examined were affected by the γ- and δ-isomers of hexachloro*cyclo*hexane. Cell multiplication was most directly influenced and the effect of the inhibitor was apparently related in some way to the need of an external supply of inositol for the different organisms. The respiratory processes seemed much less sensitive; in all instances the effect of the δ-isomer was much greater than that of the γ-isomer and was much less readily overcome by additional inositol. This is in agreement with the view that the simple theory of analogue antagonism cannot be maintained, and an explanation of the inhibitory effect of both isomers has yet to be found.

Our thanks are due to Imperial Chemical Industries Ltd. for generous gifts of the isomers of hexachloro*cyclo*hexane; and to Dr H. W. Buston, at whose suggestion the work was undertaken, for helpful guidance.

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(*Received* 7 *December* 1953)

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The Uptake of Thiamine by a strain of *Staphylococcus aureus* from the Duodenum of a Case of Polyneuritis

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SUMMARY: A strain of *Staphylococcus aureus* was isolated from the duodenum of a case of idiopathic steatorrhoea with polyneuritis and evidence of thiamine deficiency. This organism in washed cell suspension in glucose and thiamine at 37° was found to remove 75 % of the thiamine present within 1½ hr. This process was much retarded by reduction of the temperature to 4° and in the absence of glucose. The thiamine which was removed was not destroyed, and death of the organisms resulted in liberation of the thiamine from the organisms into the suspending fluid.

The process may be due to an active transference across cell membranes and concentration of thiamine inside the organism. It is suggested that in the presence of an abnormal intestinal flora such a mechanism may play a part in producing clinical thiamine deficiency states.

Thiamine (vitamin B_1, aneurin) is known to be an essential growth factor for certain micro-organisms including staphylococci (Knight, 1937*a*, *b*). It is also essential to man and its deficiency produces a polyneuritis. There is some evidence to suggest that certain cases of polyneuritis in man are the result of destruction of thiamine in the intestine (Joiner, McArdle & Thompson, 1950). Such cases may be associated with gastric achlorhydria in which profound changes in the bacterial flora of the intestine have been described (Knott, 1933; Frazer, 1949*a*), though Cregan, Dunlop & Haward (1953) have recently thrown doubt on these observations. It has been suggested that changes in the flora of the intestine may play an important part in determining vitamin deficiencies in the host (Frazer, 1949*b*).

These considerations led us to investigate intestinal organisms from a case of polyneuritis of probable thiamine deficiency origin and to make a study of the behaviour of these organisms with respect to thiamine.

MATERIALS

The source of micro-organisms used in this investigation was a patient with polyneuritis, a man of 75 years who had for 6 years suffered from looseness of the bowels and a macrocytic anaemia and had recently become bedridden on account of a severe polyneuritis. Investigation revealed steatorrhoea, achlorhydria and macrocytic anaemia; blood pyruvate examination by Prof. R. H. S. Thompson showed evidence of impaired pyruvate metabolism, indicating deranged thiamine metabolism.

A diagnosis of idiopathic steatorrhoea and progressive polyneuritis due to thiamine deficiency was made by Dr W. N. Mann. The patient's condition deteriorated and he died during a respiratory infection. The findings at autopsy were in keeping with the clinical diagnosis.

During life gastric contents were obtained from the patient by means of aspiration through a sterile gastric tube. Among the organisms isolated was a strain of *Staphylococcus aureus*. 10 hr. after death fluid was obtained by aseptic aspiration of the duodenum. From this fluid among other organisms *Staph. aureus* was again isolated.

METHODS

The uptake or destruction of thiamine was studied by making thick washed cell suspensions of the organisms in a solution containing thiamine and glucose and at intervals of time centrifuging the organisms and estimating the thiamine remaining in the supernatant fluid. In preliminary experiments the thiochrome technique was used for estimating thiamine, derived from the methods described by Mawson & Thompson (1948). A considerable amount of thiamine was found to disappear in the presence of the *Staph. aureus* suspension.

For quantitative work the microbiological technique of thiamine estimation was used. This method is not subject to the errors resulting from organic substances which may interfere with the thiochrome fluorescence. Washed cell suspensions of the strain of *Staph. aureus* were made by inoculating a loopful of the organisms from stock culture on blood agar into 5 ml. of nutrient broth, incubating for 4 hr. and inoculating the whole of this culture on Roux bottles containing nutrient agar and incubating at 37° for 18 hr. The profuse growth obtained was washed off with normal saline and the organisms were washed twice in saline, resuspended in distilled water and the suspensions were standardized turbidimetrically. These suspensions were made up to contain glucose 0·01 M, thiamine 4 μg./ml. and *Staph. aureus* in a concentration of 7·5 mg. dry weight per ml. suspension; they were kept at 37° and sampled at various times as follows. Samples were removed and pipetted into centrifuge tubes standing in iced water. The temperature of the suspension was brought rapidly to 7°. The tubes were centrifuged for 15 min. Some of the clear supernatant fluid was taken, adjusted with acetate buffer to pH 4·8 and thiamine estimations of this solution were made by a plate method of microbiological assay using *Lactobacillus fermentum* P 36, modified from the techniques described by Bacharach & Cuthbertson (1948), Jones & Morris (1949), and the operational techniques of Glaxo Laboratories, Middlesex.

Samples were taken at once (0 hr.) and after $1\frac{1}{2}$ hr. incubation at 37°. It will be noted that since the process of separation of the supernatant fluid took about 20 min. this interval of time should be added to the sampling times mentioned.

RESULTS

Disappearance of thiamine

In the presence of 0·01 M-glucose and this strain of *Staph. aureus*, there was found to be a considerable disappearance of thiamine. In the $1\frac{1}{2}$ hr. sample of supernatant fluid the thiamine had fallen to a quarter of the concentration present in the original solution of thiamine. Individual results are shown in Table 1.

The initial concentration of thiamine in the suspension was 4 μg./ml. as weighed out. In many experiments the accuracy of this figure was checked by microbiological assay.

Table 1. *Disappearance of thiamine (μg./ml.) in supernatants of suspensions of* Staph. aureus *in* 0·01 M-*glucose at* 37°

Initial conc. of thiamine	0 hr.	1½ hr.
4·0	3·6	1·1
4·0	3·0	1·3
4·0	1·9	0·5
4·0	1·7	0·5
4·0	1·7	0·4
4·0	1·6	0·9
4·0	3·1	1·5
4·0	3·7	1·2
4·0	2·7	1·4
4·0	1·7	0·8
4·0	2·4	0·7
4·0	1·7	0·8
4·0	3·4	1·1
4·0	2·7	1·2
4·5*	3·0	1·2
3·8*	2·1	0·5
Av. 4·0	2·5	0·9

* Initial concentrations of thiamine before addition of organisms checked by microbiological assay are marked in the tables with an asterisk.

The average value in twenty-two experiments in which the 4 μg. thiamine/ml. as weighed was checked by assay of the solution used was 3·8 μg./ml. This average was very near to the expected amount as weighed out. Bacharach & Cuthbertson (1948) state that the fiducial limits of the method ($P = 0·05$) are 79–133 %.

In the presence of the bacterial suspension, the 0 hr. values were well below the amount of thiamine actually put in (4 μg./ml.). The average reading in sixteen experiments was 2·5 μg./ml. It must be pointed out that whilst these experiments have been labelled '0 hr.' for convenience, this refers to time of sampling: there was in fact a 20 min. interval between making up the suspensions and the separation of the supernatant fluid from the organisms in the centrifuge, so that disappearance of thiamine must be expected during this period.

At 1½ hr. the mean value for the thiamine in the supernatant fluid in sixteen experiments was 0·9 μg./ml. This figure was quite outside the range of error of the experimental method and must be interpreted as a real fall in the concentration of the thiamine in the supernatant fluid. The difference between the mean value of the 1½ hr. readings and the mean value of the initial readings determined experimentally in 18 experiments was highly significant (differentia = $> 4 \times$ S.E.D.).

The period over which this disappearance of thiamine occurred was investigated in greater detail. Table 2 shows the results of a typical

Table 2. *Disappearance of thiamine (µg./ml.) in supernatants of suspensions of* Staph. aureus *in 0·01 M-glucose at 37°*

Initial concn. of thiamine	0 hr.	½ hr.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.
3·8*	2·6	1·3	1·1	0·9	0·9	0·8	1·1

experiment. It can be seen that the thiamine concentration fell to its lowest level in 1½–2½ hr. and thereafter increased again. The later increase is better shown in Fig. 1. That this disappearance of thiamine was dependent upon the presence of the strain of staphylococcus in the living state was shown by the following control experiments:

(1) When 0·01 M-glucose was incubated with 4 µg. thiamine/ml. at 37° without organisms the thiamine concentration was found to be unchanged (Table 3).

Table 3. *Thiamine levels (µg./ml.) in 0·01 M-glucose at 37° in absence of organisms*

Initial concn. of thiamine	0 hr.	1½ hr.	24 hr.
4	3·4	3·4	—
	3·0	3·1	4·9
	3·2	3·3	4·1
	4·0	4·0	4·2
	4·0	4·0	3·9
	4·9	4·5	4·3
	4·0	4·0	3·9
Av. 4	3·8	3·8	4·2

(2) When a suspension consisting of the strain of staphylococcus killed by boiling for 20 min. was incubated with 4 µg. thiamine/ml. and 0·01 M-glucose at 37° there was again no significant disappearance of thiamine (Table 4).

Table 4. *Thiamine levels (µg./ml.) in supernatants of suspensions of boiled* Staph. aureus *in 0·01 M-glucose at 37°*

	Initial concn. of thiamine	0 hr.	1½ hr.	24 hr.
	4·0	3·3	3·3	3·8
	4·0	3·7	3·9	
	4·0	4·5	3·9	
	4·9*	4·1	3·2	3·2
	3·4*	3·3	3·4	
	4·0	3·7	3·3	
Av.	4·1	3·8	3·5	

Effect of temperature

Experiments performed with the staphylococcus + glucose + thiamine system at 4° instead of 37° showed that, apart from an initial fall at 0 hr., no subsequent fall in thiamine concentration occurred at 1½ hr.; see Table 5.

Table 5. *Thiamine levels (μg./ml.) in supernatants of suspensions of* Staph. aureus *in* 0·01 M-*glucose at* 4°

Initial concn. of thiamine	0 hr.	1½ hr.
3·8*	3·3	3·3
3·4*	2·7	2·7
3·4*	3·4	2·7
4·2*	2·5	3·1
4·3*	2·8	3·1
4·0*	2·9	3·6
Av. 3·9	2·9	3·1

Effect of omitting glucose

Experiments were performed using staphylococci in distilled water and 4 μg. thiamine/ml. at 37° in the absence of glucose. Results are shown in Table 6 and Fig. 1. Although in some experiments the 0 hr. readings are considerably below the values given for the initial thiamine concentration, it is clear that the average values show only a slight fall in the thiamine concentration at 0 hr. and no further fall at 1½ hr. or over the subsequent 24 hr.

Table 6. *Thiamine levels (μg./ml.) in supernatants of suspensions of* Staph. aureus *in distilled water at* 37°

Initial concn. of thiamine	0 hr.	1½ hr.
4·0	2·3	2·5
4·0	2·5	2·5
4·9*	3·1	3·4
5·0*	4·1	3·9
3·0*	3·3	2·4
3·0*	2·5	3·0
3·0*	2·7	2·5
3·9*	3·9	4·2
3·9*	4·0	4·2
4·0*	3·9	3·4
Av. 3·9	2·3	3·2

Effect of continued incubation

Estimations of the thiamine in the supernatant from the thiamine + glucose + staphylococcus suspension at 37° over periods of 24–48 hr. were made (Table 7). Results of frequent estimations made over 24 hr. are shown in Fig. 1. The results of control experiments using boiled staphylococci and live staphylo-

Table 7. *Thiamine levels (μg./ml.) in supernatants of suspensions of* Staph. aureus *in* 0·01 M-*glucose at* 37° *over* 48 *hr.*

Initial concn. of thiamine	0 hr.	1½ hr.	24 hr.	48 hr.
4	1·7	0·5	2	2·1
	1·7	0·8	2·4	3·6
	1·7	0·6	2·8	2·7

cocci in distilled water are also shown. Viable counts upon the glucose and distilled water suspensions are also given.

From these it will be noted that after the initial fall in thiamine levels in the first 1½ hr. there was a gradual rise to a steady level after 20 hr. and thereafter the level remained constant. Thus, after falling to a value of 0·6 μg./ml. at 1½ hr. there was a rise to 2·9 μg./ml. at 20 and 24 hr. (Fig. 1). No such rise in the thiamine occurred with the staphylococci suspended in distilled water.

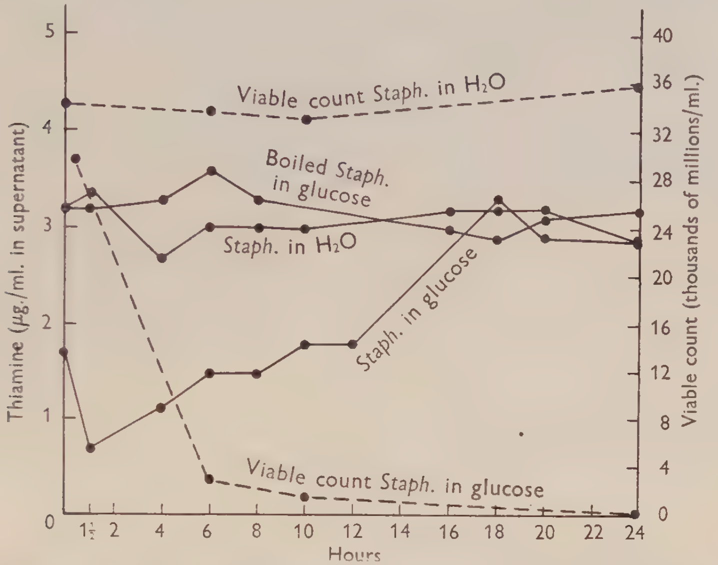

Fig. 1. Relation between viable counts and concentrations of thiamine (μg./ml.) in supernatants of suspensions of *Staph. aureus* in 0·01 M-glucose and in distilled water over 24 hr. Control of boiled *Staph. aureus* in 0·01 M-glucose containing no viable organisms.

Viable counts showed that in glucose at 37° there was a rapid fall in the number of viable organisms: the number at 6 hr. was only one-sixth of that at 1 hr. This is in contrast to the comparatively constant viable count over 24 hr. in water at 37°. In other experiments using the glucose + staphylococcus system at 4° the viable counts were also found to be constant. The fall in viable counts in glucose at 37° was associated with a fall in the pH value of the suspension from 6·8 at 0 hr. to 4·4 at 4 hr. Thereafter the pH value remained constant at 4·4.

Effect of boiling

The viable counts suggested that death of the organisms might be associated with a reappearance of thiamine. Experiments in which the organisms in the staphylococcus + thiamine + glucose suspension were killed by boiling were therefore performed.

In one experiment the thiamine in the supernatant fluid was estimated at 0 and 1½ hr. and a sample of the 1½ hr. suspension was boiled for 20 min., centrifuged and the supernatant fluid assayed for thiamine. Results are shown in Table 8.

Table 8. *Table showing return of thiamine (µg./ml.) to supernatant following boiling of suspensions of live* Staph. aureus *in* 0·01 M-*glucose at 37° after 1½ hr.*

	Live organisms	Boiled organisms
0 hr.	3·4	4·5
1½ hr.	0·1	3·9
1½ hr suspension boiled for 20 min.	4·8	3·8

Initial thiamine concn. = 4 µg./ml.

It is seen that boiling resulted in a return of thiamine in the supernatant fluid to approximately its initial value of 4 µg./ml.

Effect of Cetrimide

Similar results were obtained using Cetrimide in place of boiling. In these experiments the microbiological assay could not be used, for the Cetrimide would interfere with the growth of the test organism so that the thiochrome method had to be used. These experiments showed that much of the thiamine which had been removed from the 1½ hr. sample could be recovered by adding 0·25% Cetrimide to the 1½ hr. suspension and separating the supernatant fluid, which showed about as much thiamine as in the control solution.

Experiments with other organisms

A few experiments were performed with strains of Oxford staphylococcus, *Escherichia coli*, and *Proteus vulgaris*. These indicated that, whilst the property of thiamine removal is probably not confined to the strain of *Staph. aureus* isolated in the present work, it is very much less well marked in these other organisms.

DISCUSSION

These observations show that in the presence of the staphylococcus and glucose at 37° there was a considerable fall in the concentration of thiamine in the solution after 1½ hr. This did not take place in the absence of organisms or in the presence of organisms previously killed by boiling. The process is therefore dependent upon the presence of living organisms. The fall in the concentration of thiamine is the result of removal of thiamine by the organism rather than its destruction, for on continued incubation over 24 hr. much of the thiamine reappears. The thiamine is in some way taken up by the living organisms and subsequently released. Study of the relation between this liberation of thiamine and fall in the viable count (Fig. 1) and experiments in which organisms were killed by boiling or the action of Cetrimide suggest that whilst living organisms are essential for the removal of thiamine its subsequent liberation is related to the death of the organism.

The fall in pH value which occurs during the incubation of the staphylococcus + glucose + thiamine system was not thought to be a primary factor in the change in thiamine concentration for whilst the pH fell to its lowest value in 4 hr. there was, during this time, an initial fall and subsequent rise in thiamine concentration, and this rise continued over the next 18 hr. in the presence of a persistent low pH. Detailed investigation of the effect of pH was rendered difficult by the fact that the addition of acetate or phosphate buffers irrespective of their pH produced an effect rather like that of Cetrimide in releasing thiamine from the organisms.

In the absence of glucose there was less removal of thiamine by the organisms in the 0 hr. sample than in the presence of glucose (a removal of only 0·7 μg./ml. as compared with that of 1·5 μg./ml.) and in the absence of glucose no further removal of thiamine occurred on continued incubation. This indicates that glucose is necessary for continued removal of thiamine after the time of separation of the 0 hr. sample (about 20 min. after making up the suspension), and that it accelerates the removal of thiamine before this time. It is possible therefore that the initial removal of thiamine reflected in the low 0 hr. values may in part be due to a mechanism different from that responsible for the later removal of thiamine.

When the temperature of the glucose suspension was reduced to 4° the activity was much curtailed.

The behaviour of the process in respect to glucose and temperature suggests that there is an active removal of thiamine from the surrounding fluid by the organisms in a viable state. Such a process resembles in many features that demonstrated by Gale (1947) for glutamic acid. He showed that glutamic acid is removed from the external environment and concentrated inside *Streptococcus faecalis* in washed cell suspension in the presence of fermentable glucose by an active process of transference through the cell membrane. The process was reversed by damaging the cell membrane by boiling or by Cetrimide. However, whatever may be the explanation, the uptake of thiamine under the conditions described here does appear to be an established fact.

Destruction of thiamine by yeasts *in vitro* has been demonstrated (Lewis, 1944) and there is evidence that such a process may occur in the intestine when live yeast is fed to man by mouth (Williamson & Parsons, 1945). Ellinger & Benesch (1945) showed *in vitro* that mixed organisms from the colon of man can destroy nicotinic acid. Our experiments have revealed another phenomenon in which a vitamin is removed from the environment and stored rather than destroyed by the living organism. This property may be of biological significance to the organism, for if an organism is thiamine dependent and most strains of staphylococcus are (Knight, 1937*a*, *b*), then it is clearly of value if the organism is able to take up thiamine, thus securing a ready supply of the vitamin for use should it be faced with circumstances in which a suboptimal concentration of the vitamin occurs.

Could the presence of this organism have played a part in determining thiamine deficiency in the host? We have no direct proof, but it may be observed that if there were a heavy infection with this organism and the phenomenon of thiamine uptake were to occur in the intestine it is not unreasonable to suppose that the thiamine in the diet (normally a few milligrams per day) might be taken up by the organism, and since in the faeces there is a considerable weight of living organisms, the thiamine thus captured might entirely escape absorption by the host.

We should like to thank Prof. R. H. S. Thompson for stimulating our interest in the problem and for his help throughout the investigation, and Dr W. F. J. Cuthbertson, Glaxo Laboratories, for his ready advice on the techniques and problems of thiamine assay. We are also grateful to Dr W. N. Mann for making it possible for us to investigate his patient.

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(*Received* 14 *December* 1953)

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Site of action of Polymyxin on *Pseudomonas aeruginosa*: Antagonism by Cations

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SUMMARY: *N*-tolyl-α-naphthylamine-8-sulphonic acid forms conjugates with protein which fluoresce when excited by ultraviolet light. When washed cell suspensions of *Pseudomonas aeruginosa* are treated with *N*-tolyl-α-naphthylamine-8-sulphonic acid no fluorescence develops, but when polymyxin is also added, fluorescence develops, thus demonstrating that the dye can penetrate to protein-containing portions of the cells. This technique has been used to study the competition between polymyxin and certain cations for sites on the cells. From a comparison of the affinities of these cations for the polymyxin-combining groups of the cells with their ability to reverse the charge on certain colloids it is suggested that the polymyxin-combining loci of the cells may be polyphosphates.

The addition of polymyxin to a washed cell suspension of a strain of *Pseudomonas aeruginosa* results in a leakage from the cells of pentose, phosphate and materials which have an absorption maximum at 260 mμ. (Newton, 1953*a*). It has been suggested that the bactericidal activity of polymyxin may be due to its ability to combine with certain chemical groups on or just below the cell surface, with a resultant disorganization of a cell membrane or osmotic barrier. Similar findings have been described for other organisms (Few & Schulman, 1953). In a preliminary communication Newton (1953*b*) showed that cells could be protected against the bactericidal action of polymyxin by cations whose presence prevented the absorption of the antibiotic by the cells. There was no evidence for the formation of a polymyxin-metal ion complex, and it was suggested (Newton, 1953*c*) that there is a competition between polymyxin and cations for sites on or in the cells. The present paper gives a more detailed account of this competition from which some indication of the nature of the 'polymyxin-combining' groups of the cells can be obtained.

METHODS

Organism. The strain of *Ps. aeruginosa* previously described (Newton, 1953*a*) was used.

Medium, conditions of culture and harvesting. The organism was grown for 15 hr. at 30° in tryptic digest of casein which contained the equivalent of 3% (w/v) casein, and had an initial pH value of 7·4–7·6. Roux bottles, each containing 150 ml. medium, were inoculated with 2 ml. of an overnight culture in the same medium. Cells were harvested by centrifugation, washed twice in 1% (w/v) sodium chloride and finally suspended in saline of the same strength to give a suspension of *c.* 10 mg. dry-wt. cells/ml. (Newton, 1953*a*). Dry weights were determined turbidimetrically on a Hilger absorptiometer previously calibrated in terms of the organism used.

The use of N-*tolyl-α-naphthylamine-8-sulphonic acid to detect a change in permeability of washed cells.* Aqueous solutions of *N*-tolyl-α-naphthylamine-8-sulphonic acid (TNS) do not fluoresce when excited by ultraviolet light; in the presence of protein the dye combines with negatively charged groups, and the conjugate fluoresces strongly when excited by light of wavelength 436 mμ. (Weber & Laurence, 1954). When washed cells of *Ps. aeruginosa* were suspended in dilute solutions of this dye no fluorescence was observed, indicating that there were no groups on the surface of the cells with which the dye could combine. The addition of polymyxin to such cell suspensions resulted in an immediate fluorescence; presumably the antibiotic caused an alteration of cell permeability and allowed the dye to penetrate into the cell where it combined with cell protein. The measurement of the intensity of fluorescence of polymyxin-treated cell suspensions in the presence of TNS has provided a means of determining the rate of combination of polymyxin with cells in the presence of certain cations.

Measurement of intensity of fluorescence. Intensities of fluorescence of treated cell suspensions were measured by comparison with a standard fluorescent solution with a modified Pulfrich photometer. The apparatus described by Weber (1952) was modified so that measurements could be made under conditions of constant temperature. The exciting light from a mercury arc was filtered through a 5850 Corning glass filter and the fluorescence observed through a 335 Corning glass filter.

Standard solution of N-*tolyl-α-naphthylamine-8-sulphonic acid* (TNS). A 10^{-3}M solution of TNS in 1% (w/v) sodium chloride was used in all experiments. A trace of sodium bicarbonate was added to give a final pH value of 7.

Standard fluorescent solution. A fluorescent solution for use as a standard in the Pulfrich photometer was prepared by adding 2 ml. standard TNS solution (≡2 μmole) to 2·5 ml. of a 0·1% (w/v) solution of crystalline bovine serum albumin (Armour Laboratories, batch no. 17150) in 0·01 M-phosphate buffer, (pH 6·3). This solution was made up to 10 ml. with 1% (w/v) sodium chloride.

Treatment of cell suspensions. Washed cell suspension (0·1 ml.; 10 mg. dry-wt. cells/ml.) was added to (7·9–x) ml. 1% (w/v) sodium chloride to which 2 ml. standard TNS solution had been added; x ml. polymyxin (200 μg./ml.) were then added and the intensity of fluorescence measured after 1 min. When the effect of cations on the development of fluorescence was studied the cations were added to the cell suspension 5 min. before the addition of polymyxin, and the intensity of fluorescence was measured at intervals, commencing 1 min. after the addition of polymyxin. In some experiments cells were pretreated by suspending in uranyl chloride solutions, followed after 5 min. by washing with 1% (w/v) NaCl before treatment with polymyxin in the presence of TNS as described above.

RESULTS

Relationship between polymyxin concentration and intensity of fluorescence

The intensity of fluorescence of cell suspensions in the presence of 2 μmole TNS increased linearly with increasing polymyxin concentration (Fig. 1). Maximum fluorescence was obtained with 120 μg. polymyxin/mg. dry-wt. cells; increasing the concentration of TNS did not increase the maximum intensity of fluorescence. When cell suspensions were heated to 90° for 10 min. and then cooled, addition of TNS resulted in an immediate fluorescence which was of the same intensity as that obtained with unheated suspensions treated with 120 μg. polymyxin/mg. dry-wt. cells.

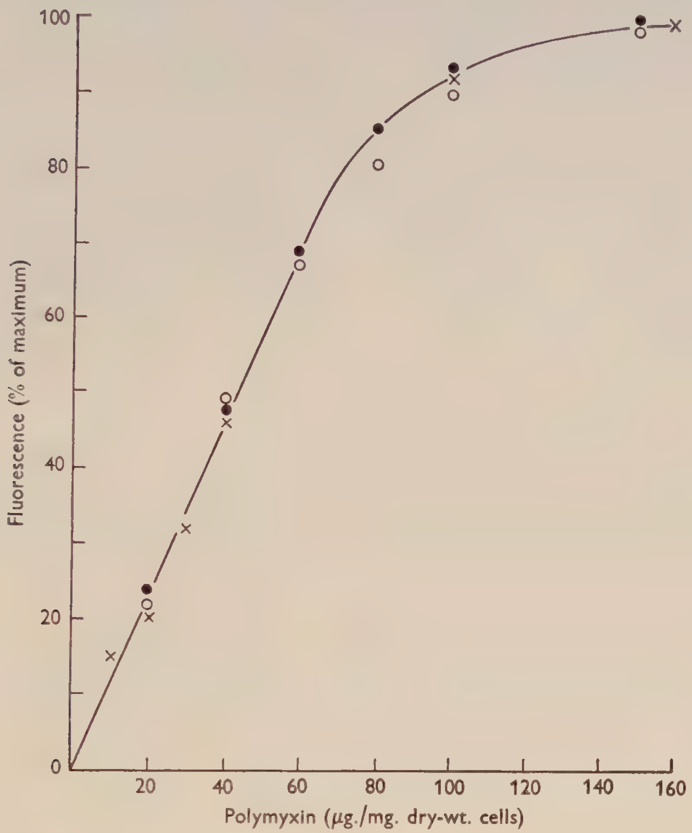


Fig. 1. Relationship between intensity of fluorescence and polymyxin concentration. Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl+2 μmole *N*-tolyl-α-naphthylamine-8-sulphonic acid+polymyxin; final volume 10 ml. Intensity of fluorescence measured 1 min. after the addition of polymyxin.

Competition between polymyxin and cations

When excess polymyxin (150 μg./mg. dry-wt. cells) was added to cells suspended in 1% (w/v) saline + 2 μmole TNS, fluorescence developed at a rate too rapid to be measured so that for experimental purposes maximum fluorescence resulted immediately. When bivalent cations were added to the cell suspension before the polymyxin the fluorescence of the suspension increased gradually, at a rate dependent on the cation concentration. Fig. 2*a* shows the effect of magnesium concentration on the rate of increase of fluorescence; there is a linear relationship between rate and concentration over the range 5–20 μmole Mg/mg. dry-wt. cells (Fig. 2*b*).

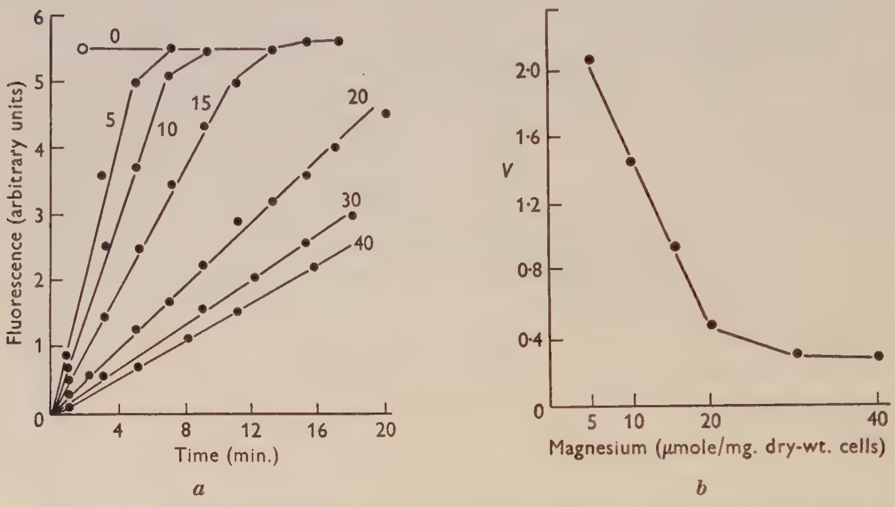


Fig. 2*a*, *b*. **Effect of magnesium concentration on the rate of increase in intensity of fluorescence of cell suspensions in presence of *N*-tolyl-α-naphthylamine-8-sulphonic acid and polymyxin. Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl + 2 μmole TNS + magnesium chloride; 150 μg. polymyxin added 5 min. after the addition of magnesium and intensity of fluorescence measured after 1 min. and at intervals thereafter. *a*: intensity of fluorescence plotted against time, figures on graph indicate magnesium concentration (μmole) added to cell suspensions. *b*: rate of increase in fluorescence (*V*) plotted against magnesium concentration.**

A number of uni-, bi- and tervalent cations were tested (Fig. 3*a*, *b*). Univalent ions (Na$^+$, K$^+$, Li$^+$ and NH$_4^+$) did not compete with polymyxin for sites on the cells, with the result that addition of polymyxin to cells suspended in the presence of these cations and TNS produced 'immediate' maximum fluorescence. Four bivalent cations were tested at a concentration of 10 μmole/mg. dry-wt. cells; it was found that the rate of increase in fluorescence varied with the different cations in the order Mg^{++} > Sr^{++} > Ca^{++} > Ba^{++}. The rate in each case has been taken as a measure of the affinity of a particular cation for the polymyxin-combining groups of the cells, i.e. the greater the degree of dissociation of the cation-cell complex the greater the rate of increase in fluorescence on addition of polymyxin. Tervalent cations were effective at lower concentrations (0·2μmole/mg. dry-wt. cells) than bivalent cations; cerium was found to have a greater affinity than lanthanum for the polymyxin-combining groups of the cells. In all experiments chlorides of metals were used (except for cerium, which was used as sulphate), but it was found that the nature of the anion did not affect the result.

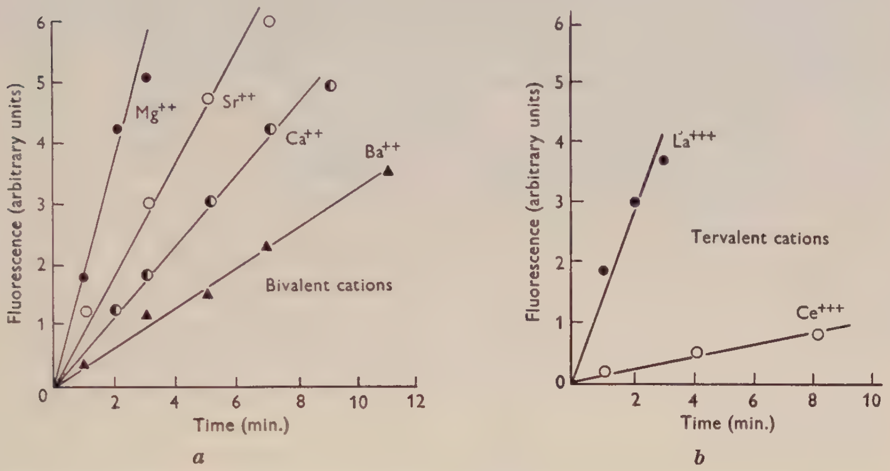


Fig. 3*a*, *b*. **Effect of bi- and tervalent cations on the rate of increase in intensity of fluorescence of cell suspensions in the presence of *N*-tolyl-α-naphthylamine-8-sulphonic acid and polymyxin. Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl + 2 μmoles TNS + cations (bivalent; 10 μmole/mg. dry-wt. cells; tervalent; 0·2 μmole/mg. dry-wt. cells). Polymyxin (150 μg.) was added 5 min. after addition of cations and the intensity of fluorescence measured after 1 min. and at intervals thereafter.**

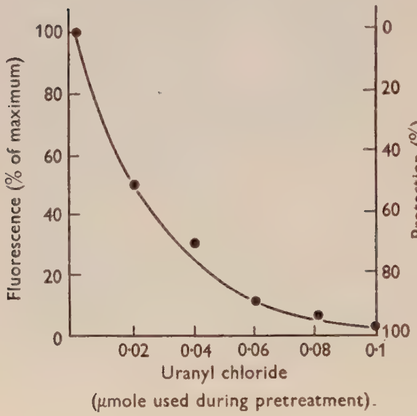
Protection of cells against polymyxin by uranyl ions (UO_2^{++})

In contrast to the results obtained with other cations the uranyl complex with the cells appeared to be relatively undissociated so that polymyxin did not combine with uranyl-treated cells even after long exposures to the antibiotic. Fig. 4*a* shows the relationship between the amount of uranyl chloride used to treat a suspension containing 1 mg. dry-wt. cells and the percentage protection against polymyxin. Uranyl-treated cells were washed repeatedly with 1% (w/v) sodium chloride without any decrease in the percentage protection against polymyxin. Uranyl ions could be removed from the cells by washing with polyphosphates, sodium hexametaphosphate being the most effective. The protection resulting from treatment of cells with 0·1 μmole uranyl chloride/mg. dry-wt. cells was 50% annulled by 2 μmole sodium hexametaphosphate and 100% annulled by 10 μmole (Fig. 4*b*). Table 1 shows the relative powers of other polyphosphates to annul the protective action of uranyl ions. Hexose diphosphate, sodium dihydrogen phosphate and sodium citrate had no annulling action at the concentrations tested.

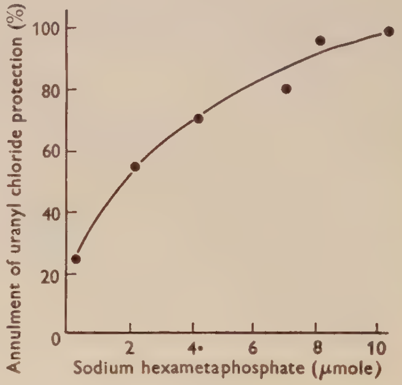
Table 1. *Annulment of uranyl chloride protection*

Washed cells (1 mg. dry wt.) suspended in 1 % (w/v) NaCl+0·1 µmole UO_2Cl_2 for 5 min., centrifuged, washed with 1 % NaCl and treated with the substances shown below for 5 min., then centrifuged, washed with 1 % NaCl and resuspended in 1 % NaCl+2 µmoles *N*-tolyl-α-naphthylamine-8-sulphonic acid, and finally 150 µg. polymyxin added. Intensity of fluorescence was measured after 1 min. Results expressed as % of maximum fluorescence in absence of cations (i.e. % annulment of uranyl chloride protection).

	Concentration (µmole/mg. dry-wt. cells)	% annulment of UO_2Cl_2 protection
Sodium hexametaphosphate	5	75
Sodium pyrophosphate	10	74
Adenosine triphosphate	100	78
Hexose diphosphate	100	0
Sodium dihydrogen phosphate	1000	0
Sodium citrate	1000	0



a



b

Fig. 4*a*. Protection of cells against polymyxin by pretreatment with uranyl chloride. Washed cells (1 mg. dry wt.) suspended in 1 % (w/v) NaCl, treated with UO_2Cl_2 at concentrations shown for 5 min., centrifuged, washed once with 1 % NaCl, resuspended in 1 % NaCl+2 µmole *N*-tolyl-α-naphthylamine-8-sulphonic acid and 150 µg. polymyxin added. Intensity of fluorescence measured after 1 min.

Fig. 4*b*. Reversal of uranyl chloride protection by sodium hexametaphosphate. Washed cells (1 mg. dry wt.) suspended in 1 % (w/v) NaCl+0·1 µmole UO_2Cl_2 for 5 min., centrifuged washed once with 1 % NaCl and suspended in sodium hexametaphosphate, at the concentrations shown, for 5 min., centrifuged, washed with 1 % NaCl, resuspended in 1 % NaCl+2 µmole *N*-tolyl-α-naphthylamine-8-sulphonic acid and 150 µg. polymyxin added 1 min. before intensity of fluorescence was measured.

DISCUSSION

The leakage of soluble constituents from washed cells of *Ps. aeruginosa* which results from the addition of polymyxin suggested that the bactericidal activity of this antibiotic might be due to its ability to disorganize the osmotic barrier of a cell by chemical combination with some of its components (Newton, 1953*a*). The penetration of *N*-tolyl-α-naphthylamine-8-sulphonic acid into polymyxin-treated cells has provided a more direct demonstration of the immediate permeability change which occurs in the presence of this antibiotic.

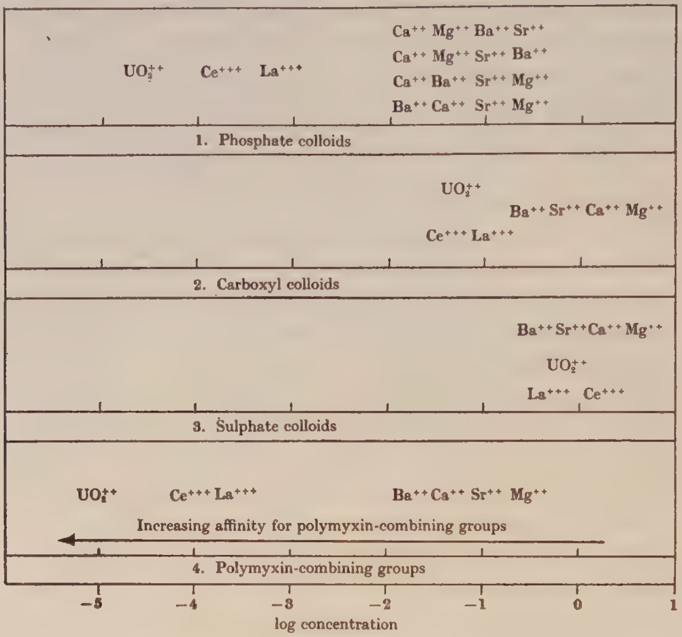
Certain antibiotics appear to have an avidity for cations (Albert, 1953), the annulment of aureomycin inhibition of a cell-free nitro-reductase system by manganese being attributed to the formation of a chelate (Saz & Slie, 1953). There is no evidence for chelation between polymyxin and cations (Newton, 1953*b*); the results presented in the present paper indicate that the antagonism of this antibiotic by cations is due to a competition between the cations and polymyxin for sites on the cells. In this respect polymyxin appears to resemble cationic detergents; a number of workers have described competition between non-toxic cations and toxic surface active cations (Valko & DuBois, 1944; Ridenour & Armbrustin, 1948; Massart, 1952).

It is of interest to compare the affinities of bi- and tervalent cations for the polymyxin-combining groups of the cells with the ability of these ions to reverse the charge on certain types of colloids. Bungenburg de Jong (1949) studied the reversal of charge on certain types of colloids by series of cations. He found that with 'phosphate colloids' (i.e. 'colloids' with ester-phosphate as ionogenic groups, e.g. egg lecithin, soya-bean phosphatides, thymus and yeast nucleates) increasing the ion radius of tervalent cations ($Ce^{+++} \rightarrow La^{+++}$) increased the reversal of charge concentration. This did not apply to bivalent cations in which the order of efficacy of a number of cations differed irregularly with the particular 'phosphate colloid' studied. However, the UO_2^{++} ion though bivalent showed reversal of charge at extraordinarily low concentration, less than that of tervalent ions. In the case of 'carboxyl colloids' (i.e. 'colloids' with carboxyl groups as ionogenic groups, e.g. Na arabinate, Na pectinate and Na pectate) increasing the ion radius of bivalent cations decreased the reversal of charge concentration ($UO_2^{++} < Ba^{++} < Sr^{++} < Ca^{++} < Mg^{++}$), UO_2^{++} occupying no exceptional position: only in the case of tervalent ions was the sequence still the same as for 'phosphate colloids' ($Ce^{+++} < La^{+++}$). For 'sulphate colloids' (i.e. 'colloids' with ester-sulphate as ionogenic groups, e.g. Na-agar and K-chondroitin sulphate), increasing the ion radius of bi- and tervalent ions decreased the reversal of charge concentration, Ce^{+++}, La^{+++} and UO_2^{++} being effective in nearly the same concentration as the bivalent ions. Table 2 sets out the various relationships diagrammatically and shows that there is a close relationship between the cation sequence for the reversal of charge on 'phosphate colloid'' and the affinities of these ions for the polymyxin-combining groups of washed cells of *Ps. aeruginosa*. It would thus appear that polymyxin may combine with phosphate groups near the cell surface.

Rothstein & Larrabee (1948) found that yeast cells could bind UO_2^{++} ions and presented evidence for the formation of a highly undissociated complex containing uranium at the cell surface. In a later paper (Rothstein & Meier, 1951) it was suggested that the uranium complexing groups of the cell surface were polymers of phosphate. The fact that UO_2^{++} protection of washed cells of *Ps. aeruginosa* could be annulled by polymerized phosphates but not by inorganic phosphate suggests that the polymyxin-combining loci of the cell surface may also be polyphosphates.

Table 2. *Comparison of specific cation sequences for reversal of charge on certain types of colloids (Bungenburg de Jong, 1949) with the affinities of these cations for the polymyxin combining groups on washed cells of* Ps. aeruginosa

Cation sequences 1, 2 and 3 summarize the results of Bungenburg de Jong (1949) and show the concentrations at which cations reverse the charge on certain types of colloids (1, 'phosphate colloids'; 2, 'carboxyl colloids' and 3, 'sulphate colloids'). Cation sequence 4 summarizes the results recorded in the present paper and shows the concentrations at which cations protect washed cells of *Ps. aeruginosa* against the bactericidal activity of polymyxin. There is a close relationship between the cation sequence of the reversal of charge on 'phosphate colloids', and the affinity of these ions for the 'polymyxin-combining groups' of washed cells of *Ps. aeruginosa.*



The author wishes to thank Dr E. F. Gale, F.R.S., for his continuous interest and encouragement. He is also grateful to Dr G. Weber for many helpful discussions and for a gift of *N*-tolyl-α-naphthylamine-8-sulphonic acid, and to Chas. Pfizer and Co. Inc. for a supply of polymyxin B sulphate.

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The Use of Suspensions of Isolated Cells for the Study of the factors affecting the Multiplication of Fowl-plague Virus

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SUMMARY: The multiplication of fowl-plague virus was studied in cultures prepared with suspensions of isolated cells of chick embryo tissues. Under favourable conditions, virus infectivity increased exponentially for 20 hr., after a latent period lasting 4–6 hr. Virus haemagglutinin production varied linearly with the number of cells added to the cultures, the regression line of this relationship showing a slope of approximately 1 when the cell concentration was kept constant and a slope higher than 1 when the number of cells was varied together with the cell concentration. Haemagglutinin production was decreased by increasing the size of the virus inoculum. A similar decrease was observed in cultures in which the depth of the fluid layer was increased. Cultures incubated at 32° for 2 days showed no haemagglutinin titres. The titres reached in cultures incubated at 35, 37 and 39° did not differ significantly. Cultures kept at 37° maintained their capacity to support virus multiplication at an approximately constant level for at least 3 days. At 22° and at 4° this capacity decreased progressively with time of storage, the decrease being more pronounced at the lower temperature. Virus multiplication was inhibited by horse serum.

Suspensions of isolated cells were used by Rous, McMaster & Hudack (1935) for the study of cell-virus interactions, and more recently by Dulbecco & Vogt (1953) for investigations on the growth of equine encephalomyelitis and poliomyelitis viruses. The multiplication of fowl-plague virus in cultures prepared with chick embryo cell suspensions was previously reported (Pereira, 1953). These cultures consist of a known number of washed cells suspended in a saline solution of known chemical composition and constitute a system in which some of the factors involved in virus production may be controlled. The object of the present paper is to report the results of the study of some of these factors.

MATERIALS AND METHODS

Preparation of cell suspensions. The method used for the preparation of chick embryo cell suspensions was based on the technique devised by Shannon, Earle & Waltz (1952) and modified by Dulbecco (1952). Ten-day chick embryos were collected, decapitated, washed in Gey's (Gey & Gey, 1936) balanced salt solution (BSS) and minced by means of Craigie's pressure mincer (Craigie, 1949) with a coarse plunger (1 mm. wide grooves). The minced tissue was collected in BSS (3 ml./embryo) and the suspension was left to stand for 10 min. The supernatant was removed and discarded and a 0·5% (w/v) solution of trypsin (L. Light and Co. Ltd., Colnbrook, Bucks) in BSS was added to the deposit in the proportion of 3 ml./embryo. The tube was placed in a water bath at 37° for 10 min. following which the tissue fragments were broken up by repeated sucking in and out of a syringe fitted with a lumbar-puncture needle. The suspension was then composed almost entirely of isolated cells and the remaining clumps were eliminated by straining through 100-mesh Monel wire gauze. The suspension was diluted 1/3 in BSS and then centrifuged for 5 min. at about 1000 r.p.m. The supernatant was discarded and the deposit was washed twice by re-suspension in BSS and centrifugation. The final suspension was again strained through 100-mesh wire gauze. Cell counts were made in a haemocytometer using white cell counting pipette and diluting fluid. Suspensions made in the proportion of 4 ml. BSS/10-day embryo contained about 10^7 cells/ml.

Virus. The 'Dutch' strain of fowl-plague virus was maintained by allantoic passage in 10-day embryonated eggs and the virus-containing allantoic fluids were preserved in sealed capillaries at −76°. Two batches of capillaries were used throughout these studies, both containing $10^{9 \cdot 7}$ egg infectivity doses/ml.

Virus titrations. (*a*) *Haemagglutination tests* were performed by a pattern technique using 0·2 ml. of twofold dilutions of the virus preparation to which were added equal volumes of a 0·5 % (v/v) suspension of washed chicken red cells. The results were expressed as the log of the number of agglutinating doses (AD) present in the total volume of each culture, one AD corresponding to the amount of virus capable of causing 50 % agglutination of the red cells contained in 0·2 ml. of the suspension.

(*b*) *Egg infectivity tests* were performed by the inoculation of 0·05 ml. of tenfold dilutions of the virus preparations in broth saline into the allantoic cavity of 10-day embryonated eggs. Each dilution was inoculated into four eggs and the end-point was calculated by the method of Reed & Muench (1938). The results were expressed as the log of the number of 50 % egg infectivity doses (EID 50) present in the total volume of each culture.

Preparation of cultures. The cultures were set up in $3 \times \frac{5}{8}$ in. tubes, stoppered with rubber bungs, sloped at an angle of about 5° from the horizontal and incubated at 37°. The total volume was adjusted to 1 ml. These conditions were kept constant unless otherwise specified. Penicillin and streptomycin were added to all the cultures to give a final concentration of 50 units and 50 μg./ml., respectively. In the growth-curve experiment the cultures were set up in 2 ml. ampoules which were sealed, sloped and incubated. At appropriate intervals, groups of 3 ampoules were frozen and preserved in solid CO_2. At the time of titration each culture was thawed in a water bath at 37°, its contents were lightly centrifuged and the supernatant was titrated by egg infectivity.

RESULTS

The growth curve of fowl-plague virus

The curve of infectivity production in cultures started with $10^{6 \cdot 8}$ cells and $10^{4 \cdot 7}$ EID 50 of fowl-plague virus is shown in Fig. 1. The number of EID 50 detected at time 0 corresponded to about 5 % of the amount inoculated. Additional experiments revealed that 80 % of the infectivity of the inoculum was lost as a result of the process of freezing and thawing and an additional 15% was retained in the deposit of the cultures. The infectivity was further decreased to about 1% of the inoculum after 4 hr. of incubation and then increased exponentially for 20 hr. At 40 and 48 hr. the infectivity was only slightly higher than at 24 hr. In another experiment in which cultures were tested after 2, 4 and 6 hr. of incubation the period of low infectivity was shown to last between 4 and 6 hr.

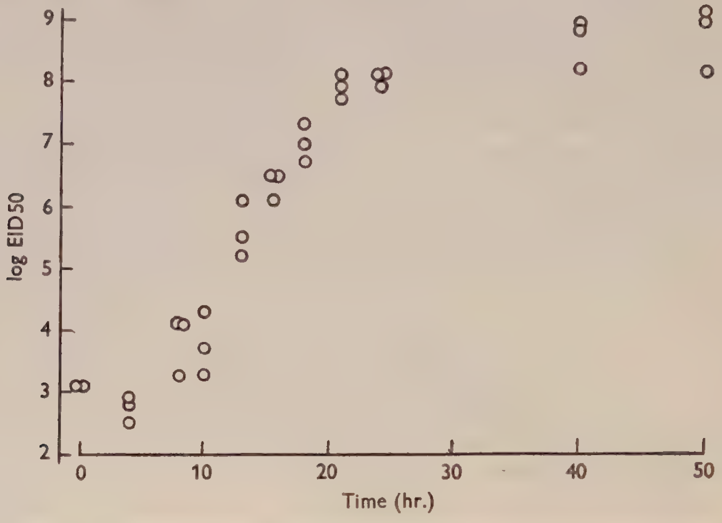

Fig. 1. Growth curve of fowl-plague virus in cell suspension cultures.

Number of cells

Two series of experiments were performed to study the relationship between the number of cells present in the cultures and the amount of virus produced. In the first series the number of cells in successive groups of tubes was varied, together with the total volume of fluid, the concentration being constant in all the tubes ($10^{6\cdot7}$ cells/ml.). In the second series the number of cells was varied, together with the cell concentration, the volume of fluid being constant in all the cultures (1 ml.). In all these experiments the total number of cells was varied in the region between 10^6 and 10^7. The virus inoculum was added to all the cultures in a final dilution of 10^{-5} ($10^{4\cdot7}$ EID50/ml.). The tubes were slanted, incubated at 37° for 2 days, following which the supernatants were titrated by haemagglutination. The results are shown in Figs. 2 and 3, in which each point corresponds to the average of four determinations. In both series a linear relationship was observed between the number of cells present in the cultures and the amount of haemagglutinin produced. The slopes of the regression lines gave values of 0·998 for the series with constant cell concentration and 1·709 for the series with varying cell concentrations. A *t*-test between the two slopes showed that they are significantly different ($t=4\cdot29$ with 51 D.F., $P<0\cdot001$). The possibility that the increased virus production in high cell concentrations might be due to a stimulation by substances derived from the embryo and improperly washed away in the process of preparation of the suspension was considered. A suspension was prepared by the usual technique except that the cells were washed by four cycles of centrifugation and resuspension instead of three. Cultures prepared with constant numbers of cells suspended in the three last supernatants obtained in the washing process and in BSS revealed no difference in virus-producing capacity.

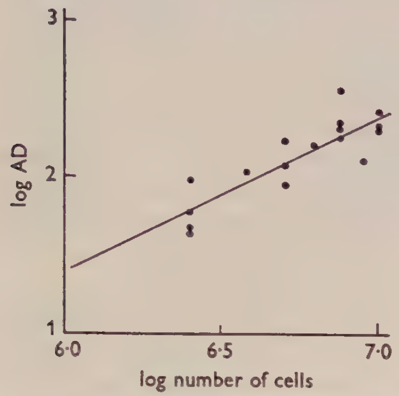

Fig. 2. Relation between number of cells and haemagglutinin production. Constant cell concentration. Varying volumes.

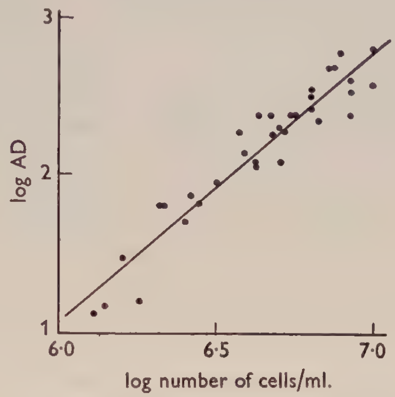

Fig. 3. Relation between number of cells and haemagglutinin production. Varying cell concentrations. Constant volume.

Size of the virus inoculum

Two groups of sixteen tubes, each containing $10^{6\cdot7}$ cells, were inoculated respectively with $10^{6\cdot7}$ and $10^{3\cdot7}$ EID50 of fowl-plague virus. The volume was adjusted to 1 ml. in all the tubes. After 2 days of incubation under standard conditions the supernatant fluid of each culture was titrated by haemagglutination. The average log of the number of AD produced per tube was calculated for each series. For the series receiving the larger virus inoculum this value was 1·83 with a standard deviation of 0·08 and for the series receiving the smaller virus inoculum it was 2·21 with a standard deviation of 0·11. A *t*-test between these two averages showed a significant difference ($t = 10\cdot21$ with 30 D.F., $P < 0\cdot001$).

Temperature of incubation

Four groups of twelve cultures were prepared, each tube containing $10^{6\cdot7}$ cells and $10^{4\cdot7}$ EID50 of fowl-plague virus. The different groups were incubated respectively at temperatures of 32, 35, 37 and 39°. After 2 days the supernatant fluid of each culture was titrated by haemagglutination and the results obtained for each group were averaged. Table 1 summarises the results. In the cultures incubated at 32° no haemagglutinating activity was detected after 2 days of incubation. The average log AD of the groups of cultures incubated at 35, 37 and 39° were not significantly different.

Table 1. *Influence of temperature of incubation on virus haemagglutinin production*

Temperature of incubation (°)	Average log AD	Standard deviation
32	$<0{\cdot}67$	—
35	2·31	0·24
37	2·24	0·14
39	2·37	0·12

Depth of fluid layer

Two groups of sixteen cultures were prepared, each tube containing $10^{6\cdot7}$ cells and $10^{3\cdot7}$ EID50 of fowl-plague virus. The first group was set up in $4 \times \frac{1}{2}$ in. tubes which were incubated upright, the fluid forming a column about 15 mm. high. The second group was set up in $3 \times \frac{3}{8}$ in. tubes which were incubated at an angle of 5° from the horizontal, the fluid column at its deepest part showing a height of about 3 mm. After 2 days incubation at 37° the supernatant fluid of each culture was titrated by haemagglutination. The average log of the number of AD produced per tube gave values of 1·59 with a standard deviation of 0·19 for the cultures with deep fluid layer and 2·21 with a standard deviation of 0·11 for the cultures with shallow fluid layer. A t-test between these two averages showed a significant difference ($t = 11{\cdot}06$ with 30 D.F., $P < 0{\cdot}001$).

Storage of cell suspension

The effect of storage at different temperatures on the capacity of the cell suspension to support virus multiplication was investigated. Thirty cultures were prepared containing $10^{6\cdot8}$ cells in a volume of 0·9 ml. Three tubes were immediately inoculated with $10^{4\cdot7}$ EID50 of fowl-plague virus in a volume of 0·1 ml. and incubated at 37°. The remaining tubes were divided into three groups which were sloped and kept at 4, 22 and 37° respectively. After 24 hr. three tubes of each group were inoculated with fowl-plague virus as above and incubated at 37°. At 48 and 72 hr. further groups of tubes were treated similarly. After addition of the virus the tubes were incubated at 37° for 2 days and then preserved at 4° until the end of the experiment, when the supernatant fluid of each culture was titrated by haemagglutination. The result is shown in Fig. 4 in which each point represents the average log AD of the three tubes of each group. The virus-producing capacity of the cultures kept at 37° remained approximately constant up to 72 hr. At 22 and at 4° the virus-producing capacity decreased progressively with the time of storage, this decrease being more pronounced at the lower temperature. In the cultures kept at 37° the cells were observed to adhere to the glass forming a rather irregular sheet containing many fibroblast-like elements.

Inhibitory action of horse serum

Six groups of four cultures were prepared containing $10^{6\cdot8}$ cells and $10^{4\cdot7}$ EID50 of fowl-plague virus. To five of the six groups unheated normal horse serum was added at concentrations varying from 2 to 10% and to the remaining group no horse serum was added. After 2 days of incubation under standard conditions the supernatant fluid of each culture was titrated by haemagglutination. The result is shown in Fig. 5 in which each point represents the average log AD of each group. These values decrease linearly with increasing concentrations of horse serum. At the end of the incubation period all the cultures showed a layer of cells adhering to the wall of the tube. In the cultures without serum this layer was very irregular and revealed on microscopic examination very few intact cells. As the serum concentration increased the cellular layer showed increasing numbers of fibroblast-like elements. Further investigation demonstrated that the factor in horse serum responsible for this inhibition resisted heating at 56° for 30 min. and was inactivated by trypsin.

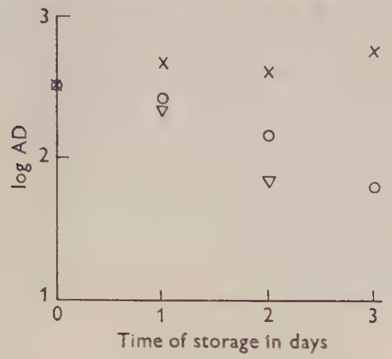

Fig. 4. Effect of storage at different temperatures on the virus-producing capacity of cell suspension cultures. ×, cultures stored at 37° before addition of virus; ○, cultures stored at 22° before addition of virus; ▽, cultures stored at 4° before addition of virus.

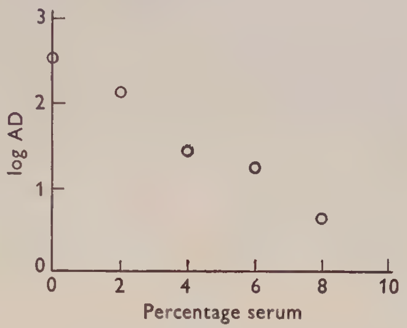

Fig. 5. Inhibition of haemagglutinin production by normal horse serum.

COMMENTS

The study of the factors influencing the growth of viruses in tissue cultures was reviewed by Robbins & Enders (1950). The interpretation of the results obtained has often been complicated by the imperfect standardization of some of the factors studied, because of technical difficulties. With the object of achieving this standardization the cultures used in the present study were started with suspensions of isolated cells. The virus of fowl-plague multiplies readily in this system reaching relatively high haemagglutination titres which proved of great value for the simultaneous titration of relatively large numbers of culture materials.

The growth of fowl-plague virus in cell suspension cultures revealed several features in common with other viruses studied under similar conditions (Crawford & Sanders, 1952; Gajdusek, 1953; Womack & Kass, 1953). A latent period of 3–5 hr. followed by a stepwise increase of infectivity was reported by Schäfer & Munk (1952) in the case of the multiplications of fowl-plague virus on the isolated chorio-allantoic membrane of chick embryos. The heterogeneous nature of the cell suspension used in the present study prevented the estimation of the multiplicity of infection and, as no attempts were made to prevent re-infection of cells by newly formed virus, no evidence for a stepwise growth cycle could be obtained. Dulbecco & Vogt (1953) described one-step growth curves of poliomyelitis and Western equine encephalomyelitis viruses in cell suspensions.

The relationship between number of cells and virus production in tissue cultures has been studied by several authors (Hallauer, 1932; Plotz, 1933; Rivers & Ward, 1933; Traub, 1933; Fox, 1947; Yanamura & Meyer, 1941; Simms & Sanders, 1942; Ledinko, Riordan & Melnick, 1952; Womack & Kass, 1953). In most of these studies the number of cells was estimated by indirect methods such as the weight, the volume or the nitrogen content of the tissue added to the cultures. The results varied for different systems, but in general it has been found that there is an optimum amount of tissue for maximal virus production. The use of suspensions of isolated cells provides particularly favourable conditions for the study of this relationship, as by this method the number of cells may be estimated by direct count and replicate cultures may be prepared. In the region covered by our experiments a linear relationship was observed between the number of cells added to the cultures and the amount of virus haemagglutinin produced. The fact that with constant cell concentrations the regression line of this relationship shows a slope of approximately 1 (0·998) indicates that under these experimental conditions the virus concentrations obtained are constant for each cell concentration and independent of the total volume of the culture. Under these conditions it may be assumed that throughout the range studied the same average amount of virus haemagglutinin was produced by individual cells. On the other hand, when the number of cells was varied, together with the cell concentration, the slope of the cell-virus regression line gave a value higher than 1 (1·709) indicating that the average amount of virus produced per cell increases with increasing cell concentrations. This fact may represent another manifestation of cellular interactions described for several tissue culture systems (Buchsbaum, 1932; Ephrussi, 1933; Bloom, 1937; Fischer & Jensen, 1946; Earle *et al.* 1951). It is possible that cellular products diffused into the medium during the period of incubation may favourably influence virus production. The low sensitivity of the haemagglutination test used for virus titrations established the lower limit of cell concentrations studied, as the virus yields in suspensions containing less than 10^6 cells/ml. do not reach haemagglutination level. Dulbecco & Vogt (1953), who used a plaque technique of titration, demonstrated the multiplication of poliomyelitis and Western equine encephalomyelitis viruses in isolated cells. These authors' techniques differed from ours in that the cells were suspended in a nutrient medium.

It should be noted that in the series of experiments in which the total volume of the cultures was varied, the depth of the fluid column was kept low by sloping the tubes during incubation. This precaution was taken in view of the observed decrease of virus yield in cultures in which the fluid column is deep. The influence of the depth of the fluid column on virus production in tissue cultures was studied by Maitland, Laing & Lyth (1932), Yanamura & Meyer (1941), Thompson & Coates (1939), and the results have varied for the different systems under study. The use of shallow fluid layers is a procedure generally adopted in tissue culture work.

The influence of the size of inoculum on virus production in tissue cultures has been studied by several authors (Fox, 1947; Yanamura & Meyer, 1941; Womack & Kass, 1953; Sanders & Jungeblut, 1942; Dulbecco & Vogt, 1953), and in general it has been observed that the final titres obtained are not greatly affected by the size of the inoculum but the time required for maximal virus production is increased when small inocula are used. Robbins, Weller & Enders (1952), when studying the propagation of poliomyelitis viruses in roller tube cultures of human tissues, obtained relatively smaller titres in cultures inoculated with large amounts of virus. A similar result was clearly demonstrated in the present investigation. Kinetic studies of virus multiplication in cultures started with varying sizes of inoculum are necessary for a better understanding of this phenomenon.

The study of the effect of storage on the capacity of cell suspensions to support virus growth revealed that this property is preserved for longer periods at 37° than at room temperature or at 4°. This result might be explained in part by the occurrence of cellular multiplication at 37°. Cultures incubated at this temperature before addition of the virus revealed after 2 days an irregular sheet of fibroblast-like cells adhering to the glass. In virus-infected cultures the number of these cells was greatly reduced. The cellular sheet of cultures incubated for 2 days in the absence of virus revealed no mitotic figures on microscopical examination after fixation and staining by haematoxylin *in situ*.

The composition of the medium was shown to have a marked effect on the appearance of the cellular sheet formed after incubation. Increasing numbers of fibroblast-like elements were observed in cultures containing increasing concentrations of horse serum. This action of horse serum was accompanied by inhibition of virus haemagglutinin production. The mechanism of this inhibition cannot be explained as due to the depressive action of horse serum on cellular physiological activities as proposed by Fox (1947) for the inhibition of the multiplication of yellow fever virus in tissue cultures.

The author wishes to thank Miss M. V. Mussett for help in the statistical analysis of the results.

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(*Received* 1 *January* 1954)

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The Inhibition of Bacterial Growth by Colloidal Heavy-metal Sulphides and by Colloidal Sulphur

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SUMMARY: Colloidal copper sulphide is inhibitory to staphylococci and a number of other Gram-positive organisms, but has little effect on Gram-negative organisms. Its formation when cystine and peptides containing cystine are autoclaved with copper is thought to be responsible for the anti-staphylococcal activity of heat-sterilized copper-containing peptone solutions. Colloidal sulphur is active against the cocci but the inhibition differs in some respects from that of copper sulphide, notably in the ease with which it can be reversed by cystine, cysteine and mercapto-acetic (thioglycollic) acid. Iron and manganese sulphides also inhibit bacterial growth and the possible significance of this inhibition on the production of anaerobic toxins is discussed.

The failure of some bacteria to grow readily from small inocula in laboratory media was shown by O'Meara & Macsween (1936, 1937) to be due to copper present as an impurity in their peptone. The present investigation was undertaken to determine the reason for this inhibitory effect of copper, particularly for *Staphylococcus aureus* which O'Meara & Macsween showed was a susceptible organism.

METHODS

Organisms used. Staphylococcus aureus CN 59 (Wellcome Bacterial Collection) was used to test for inhibition. Representative strains of other species of bacteria from the collection were used in the later stages of the work when the inhibitory factor had been identified and a survey of the sensitivity of micro-organisms to this substance was being made.

Experimental procedure. Inhibition was detected by inoculating the medium with falling tenfold dilutions of the test organism, the initial suspension having been adjusted to match Brown's opacity tube no. 3 ($1{\cdot}8 \times 10^9$ organisms/ml. for *Staph. aureus*). The dilutions were made in quarter-strength Ringer's solution, and 1 ml. of each dilution was added to 9 ml. of medium, three tubes being inoculated at each dilution over the range 10^{-1} to 10^{-9}. The full range of dilutions was not always necessary and was adjusted to suit the experiment. Growth was examined after 24 and 48 hr. of incubation.

Media. For work with acid-hydrolysed casein and also for studying the effect of copper on individual media constituents the following medium, modified from one which supported growth of a wide range of micro-organisms (Proom & Woiwod, 1949) was used:

Acid hydrolysed casein	20·0 g.	Adenine	30 μg.
Tryptophan	0·4 g.	Pyridoxin HCl	2·4 mg.
Riboflavin	400 μg.	*p*-Aminobenzoic acid	80 μg.
Calcium pantothenate	800 μg.	Nicotinic acid	1200 μg.
Biotin	8 μg.	Choline chloride	15 mg.
Thiamine	400 μg.	Pteroylglutamic acid	0·4 μg.
Uracil	30 μg.	Distilled water to 1 l.	

The casein hydrolysate was prepared by hydrolysing light white soluble casein with *c.* 6 N-hydrochloric acid, neutralizing, decolorizing with charcoal, removing calcium by the addition of phosphates and pH adjustment, and freeze-drying the final product. The growth factors were added as a stock solution. Sterilization was by filtration through Berkefeld-type candles after the pH had been adjusted to 7·4. This medium could be diluted to half strength with distilled water without affecting its ability to support adequate growth from small inocula of CN 59. Thus solutions being tested for inhibition could be added in equal volume before inoculation. Copper was added to media and to media constituents as a solution of cupric chloride ($CuCl_2 . 2H_2O$, Analar) in distilled water. Autoclaving was always for 10 min. at 10 lb./sq.in. Nutrient agar was prepared with papain digest of horse muscle together with a horse-muscle extract, and was solidified with 1·2 % South African agar (James P. Thol Ltd., 110 Fenchurch St., E.C. 3).

RESULTS

O'Meara & Macsween (1936, 1937) showed that it was necessary to heat solutions of peptones containing copper in order to produce inhibition. This observation was confirmed with a freeze-dried tryptic digest of horse muscle prepared in this laboratory. A 1 % solution of this peptone, which contained less than 0·05 mg. Cu/g., supported the growth of CN 59 whether sterilized by heat or by filtration, growth being consistently obtained at a dilution of 10^{-8} to 10^{-9} of the standard suspension of the test organism. On adding 10 mg. Cu/l. to simulate the copper level present in the solutions of peptones examined by O'Meara & Macsween and autoclaving for 10 min. at 10 lb. the peptone solution completely inhibited the growth of CN 59. The same amount of copper added as an autoclaved solution of copper chloride to a peptone solution previously sterilized by heat was without effect. Considerably higher amounts of copper were necessary to produce inhibition when the copper and peptone solutions were sterilized separately, the results in Table 1 showing that ionic copper was inhibitory to CN 59 only at a very high concentration.

Table 1. *The inhibitory effect on* Staphylococcus aureus *of copper chloride added to unheated medium*

Three tubes of medium were inoculated at each dilution of inoculum. The numbers indicate the number of tubes showing growth at each dilution. — = no tubes inoculated at these dilutions.

	Dilution of inoculum							
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Added copper (mg./l.)	Growth response at 48 hr.							
Nil	3	3	3	3	3	3	3	2
1·0	—	—	—	—	—	3	3	2
10·0	—	—	—	—	—	3	3	0
100·0	—	—	—	—	—	3	2	2
1000·0	0	0	0	0	0	0	0	0

The effect of container on the production of inhibition

It was observed in the early stages of the work that peptone solutions containing copper became inhibitory only when autoclaved in tubes or bottles plugged with cotton-wool. When screw-capped bottles were used there was no inhibition (Table 2). This effect, for which no explanation has yet been obtained, appeared to be a function of the tightness of the seal made by the rubber disk in the screw cap with the bottle top. When this seal was prevented from forming by laying a cotton thread across the top of the bottle or by using a bottle with a chipped top, no protection was obtained and the heated medium became inhibitory. Sealed ampoules were as effective as screw-capped containers in preventing inhibition and therefore protection was almost certainly due to the air-tightness of the seal made by the rubber liner in the screw cap and not to the action of some chemical in the rubber liner. Replacement of cotton-wool plugs by caps or vice versa, after autoclaving, did not affect inhibition. Inhibitory material was therefore always prepared in plugged containers, although after heating this material was usually filled aseptically into sterile 1 oz. screw-capped bottles as these were more convenient for testing.

Table 2. *Effect of heat and type of container on the inhibition of* Staphylococcus aureus *in* 1 % *solution of tryptic digest of muscle* + 10 *mg.* Cu/*l.*

Three tubes were inoculated at each dilution of inoculum. Symbols as in Table 1.

Method of sterilization and closure	Dilution of inoculum									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
	Growth response at 48 hr.									
Autoclaved (screw-capped bottles)	3	3	3	3	3	3	3	3	1	0
Autoclaved (plugged bottles)	3	0	0	0	0	0	0	0	0	0
Candled (screw-capped bottles)	3	3	3	3	3	3	3	3	1	0
Candled (plugged bottles)	3	3	3	3	3	3	3	3	0	0

The inhibitory effect of copper in partially defined medium

Tryptic digest of muscle was unsuitable for continuing the investigation because of its complex composition. The partially defined medium made from acid hydrolysed casein with added growth factors was therefore tested to see whether the inhibitory phenomena could be reproduced. When this medium, to which 10 mg. Cu/l. had been added, was autoclaved in plugged containers it became inhibitory, whereas medium to which the same amount of copper had been added as a sterile solution was not inhibitory. The protective action of the screw-capped bottle was also observed and the results resembled in every way those obtained with tryptic digest of muscle. Inhibition was produced at copper concentrations as low as 2 mg./l., but 10 mg./l. was chosen for most of the experiments as being nearer the level of copper in the peptone solutions used by O'Meara & Macsween (1936, 1937).

Autoclaving various components of casein medium with copper before mixing with the remainder showed that inhibition arose from interaction of copper with the acid-hydrolysed casein and not with the growth factors. Of the amino acids present in casein only cystine produced an inhibitory solution when autoclaved with copper, although this amino acid autoclaved alone was inactive. It was possible therefore that an oxidation or degradation product of cystine produced during autoclaving was responsible for inhibition, copper perhaps being involved as a catalyst. Cystine disulphoxide, cysteine sulphinic acid, di-β-mercapto-di-propionic acid, ethyl disulphide and cysteic acid, all possible degradation products of cystine, were tested but none was inhibitory. Attempts were therefore made to isolate the inhibitory substance in amounts sufficient for its identification.

Testing for inhibition by the dilution technique was time-consuming and a simpler test was sought. The inhibitory factor was expected to be an easily diffusible substance of comparatively low molecular weight. Plates of nutrient agar were therefore seeded with the test organism and the material under examination placed on the plates in 'fish spine' beads as used in antibiotic plate tests. Using the dilution technique it had been found that inhibitory casein hydrolysate could be diluted up to 8 times and still retain its ability to inhibit growth of CN59. It was expected that zones of inhibition would be seen surrounding fish-spine beads containing undiluted inhibitory medium. Repeated attempts to demonstrate zones were without success, although growth was inhibited directly beneath the bead. The inhibitory factor therefore appeared unable to diffuse in agar. This failure to diffuse could be explained if the substance were a colloid. To test this possibility a solution of cystine (20 mg./l.) and copper (10 mg./l. as chloride) in 0·01 M-phosphate buffer (pH 7·4) was autoclaved and the resulting inhibitory solution, which was a clear golden brown in colour, was dialysed against running tap water for 24 hr. The colour was retained by the dialysis membrane, and the residue in the sac, which was free from ninhydrin-positive substances, was still inhibitory when added to casein medium. Hence the inhibitory substance formed on autoclaving cystine with copper was colloidal and probably brown in colour.

Among the ultimate degradation products of cystine is hydrogen sulphide which is readily detected with lead acetate paper when a solution of cystine is heated. Copper present during the heating of a solution of cystine might therefore be expected to form colloidal copper sulphide, the sulphides of many heavy metals being colloidal in dilute solution. A solution of hydrogen sulphide, standardized in the usual way with iodine, was added in graded amounts to a copper chloride solution containing 10 mg. Cu/l. in an attempt to simulate the possible formation of copper sulphide from cystine. The final H_2S concentrations lay between 1 and 5 mg./l., and it was found that hydrogen sulphide was not toxic for the test organism at these concentrations. The clear brown solutions obtained matched very closely the appearance of autoclaved solutions of cystine and copper, the colour increasing in intensity with increasing hydrogen sulphide concentration. When boiled, these solutions remained clear though the colour changed to green, but they became unstable and flocculated when mixed with medium. The unheated brown solutions could be sterilized by filtration through candles, although a certain amount of coloured material was adsorbed in the process. The candled solutions were stable and inhibited the test organism when added to casein hydrolysate medium. It was concluded that copper sulphide was the inhibitory substance produced when casein hydrolysate or cystine solutions were autoclaved with copper.

To study the inhibitory effect of colloidal copper sulphide on bacteria it was necessary to prepare a sol which was reproducible, stable and free from either hydrogen sulphide or excess copper salt. After preliminary experiments the following procedure was devised. A solution of hydrogen sulphide made by passing the gas through distilled water for 15 min. was standardized with iodine and diluted to contain 3 mg. H_2S/100 ml. One hundred ml. of this solution was added slowly with stirring to 900 ml. of distilled water containing 30 mg. $CuCl_2.2H_2O$. A clear golden-brown solution was obtained which was dialysed against distilled water for 24 hr. The material retained by the dialysis membrane was sterilized by filtration through a Berkefeld-type candle. By using small candles and filtering as large a volume of solution as possible, the loss of copper on candling could be decreased to less than 30 % and the sterile solution obtained was still strongly coloured and could be diluted up to 8 times without marked loss of activity. These inhibitory solutions were standardized on their copper content which was determined by oxidizing a sample with nitric acid and determining the copper colorimetrically with sodium diethyldithiocarbamate. Copper sulphide sols prepared by this technique were stable for several weeks and were rejected when a distinct green shade became apparent. They were used for determining inhibitory levels of copper sulphide and also for investigating substances capable of abolishing the inhibition.

To find the level at which copper sulphide ceased to inhibit the test organism, dilutions of a copper sulphide sol prepared as above were made in casein hydrolysate medium. Table 3 shows that the inhibitory effect increased with increasing sulphide concentration and there was little increase in growth after 48 hr. A different result was obtained when a similar experiment was made with a sol which had been dialysed for 48 hr. instead of 24 hr. This sol had a greenish colour, indicating a change towards a larger particle size, although it was still clear and apparently stable. On incubating for several days the medium to which the sol had been added, growth equal to that of the control was obtained even with the highest copper sulphide concentration. Inhibitory action therefore decreased with increase in particle size of the colloid.

The inhibitory material formed when tryptic digest of muscle (1 %) was autoclaved with copper (10 mg./l.) was shown to be colloidal by dialysis. The inhibitory substance was therefore most probably colloidal copper sulphide. Analysis before and after dialysis showed that 74 % of the copper initially added had been immobilized on autoclaving and failed to pass through the dialysis membrane in 48 hr. A similar experiment with casein hydrolysate medium indicated a much greater loss of copper on dialysis, only 15% of the original copper remaining after 48 hr. The larger amount of copper sulphide formed in the tryptic digest was probably a reflexion of its higher cystine content. Paper chromatography showed that little free cystine was present in the tryptic digest, most of the cystine being combined in peptides. As solutions of glutathione autoclaved with copper were inhibitory to CN 59 whereas solutions of this peptide heated alone were inactive it is probable that the cystine-containing peptides in tryptic digest of muscle produced the hydrogen sulphide necessary for copper sulphide formation.

Table 3. *The inhibition of* Staphylococcus aureus *by colloidal copper sulphide added to casein hydrolysate medium*

Three tubes inoculated at each dilution of inoculum. Symbols as in previous tables.

Duration of incubation	Added copper sulphide sol (μg. Cu/ml.)	Dilution of inoculum						
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
		Growth response						
24 hr.	Nil	3	3	3	3	3	2	0
	0·34	3	3	2	0	0	0	0
	0·68	3	0	0	0	0	0	0
	1·36	0	0	0	0	0	0	0
48 hr.	Nil	3	3	3	3	3	3	1
	0·34	3	3	3	3	3	2	1
	0·68	3	3	0	0	0	0	0
	1·36	0	0	0	0	0	0	0
7 days	Nil	3	3	3	3	3	3	1
	0·34	3	3	3	3	3	3	2
	0·68	3	3	1	0	0	0	0
	1·36	2	0	0	0	0	0	0

The inhibitory effect of colloidal sulphur on the growth of Staphylococcus aureus

Whilst this work was in progress Schuhardt and co-workers (Schuhardt, Rode, Foster & Oglesby, 1949; Schuhardt, Rode & Oglesby, 1949; Schuhardt, Rode, Oglesby & Lankford, 1950) had been investigating the anti-brucella activity of heated cystine solutions. In many ways this inhibition resembled that of copper sulphide. It was shown eventually (Schuhardt, Rode, Oglesby & Lankford, 1952) that the anti-brucella factor was colloidal sulphur. The effect of sulphur on CN 59 was therefore examined to see in what respects it behaved like colloidal copper sulphide towards this organism. Autoclaved cystine solutions were non-inhibitory for CN 59 at 50 mg. cystine/l. and this organism therefore appeared less susceptible to sulphur than the *Brucella* spp. studied by Schuhardt. Autoclaved cystine solutions were found by Fildes & Richardson (1937) to be inhibitory for staphylococci. The failure to demonstrate such inhibition in the present work suggests that Fildes & Richardson were perhaps observing copper sulphide inhibition, copper being present as a contaminant in their samples of cystine. A colloidal sulphur suspension prepared by the technique of Schuhardt *et al.* (1952) when added aseptically in graded amounts to casein medium was inhibitory to CN 59. The results summarized in Table 4 show that growth was delayed, particularly at higher sulphur concentrations, but there was a tendency for inhibition to disappear on prolonged incubation. In this sulphur differed from colloidal copper sulphide.

Table 4. *The inhibition of* Staphylococcus aureus *by collodial sulphur added to casein hydrolysate medium*

Three tubes inoculated at each dilution of inoculum. Symbols as in previous tables.

		Dilution of inoculum						
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Duration of incubation	Added colloidal sulphur (μg. S/ml.)	Growth response						
24 hr.	Nil	3	3	3	3	3	2	0
	0·15	3	3	3	3	3	3	0
	0·60	0	0	0	0	0	0	0
	1·20	0	0	0	0	0	0	0
	2·00	0	0	0	0	0	0	0
48 hr.	Nil	3	3	3	3	3	3	0
	0·15	3	3	3	3	3	3	1
	0·60	3	3	3	2	3	1	0
	1·20	1	0	0	0	0	0	0
	2·00	0	0	0	0	0	0	0
7 days	Nil	3	3	3	3	3	3	0
	0·15	3	3	3	3	3	3	1
	0·60	3	3	3	3	3	3	1
	2·00	3	3	3	2	0	0	0

The reversal of copper sulphide and sulphur inhibition

Schuhardt *et al.* (1952) found a number of substances which removed the inhibitory action of sulphur for *Br. abortus*, among them cystine, mercaptoacetic acid and various inorganic sulphur compounds. Some of these substances were tested to see whether they were equally effective in preventing the inhibition of CN 59 by sulphur and also whether they effected the inhibition of this organism by colloidal copper sulphide. The colloidal sulphur and copper sulphide solutions were prepared as before. The former was opalescent and gave a strong Tyndall effect, whereas the copper sulphide was crystal clear with little Tyndall effect. Colloidal sulphur was added to casein medium to give a final concentration of 1·6 μg. S/ml. and the copper sulphide a concentration of 2·8 μg. CuS/ml. Examination for growth was made after 24 and 48 hr. incubation, casein medium being used as a control.

Cystine, cysteine and mercaptoacetic acid were without effect on copper sulphide inhibition, although all these substances overcame the inhibitory action of sulphur. On the assumption that copper sulphide might exert its inhibitory action by blocking the formation from cysteine or cystine of a simple degradation product necessary for growth, β-mercaptopropionic acid, the product of deamination of cysteine, was examined. This substance completely reversed inhibition by both sulphur and copper sulphide. With the latter the growth was coloured brown, indicating that copper sulphide was absorbed by the bacteria. Sodium sulphite also removed inhibition. As, however, it is not a possible source of sulphur for staphylococci (Fildes & Richardson, 1937) the mechanism by which it reverses inhibition is difficult to understand.

Examination of the two sols revealed large differences in particle size. Sulphur sols prepared by the method of Schuhardt *et al.* (1952) lost their inhibitory properties when passed through the coarsest grade of membrane filter (Apis Engineering Co.), indicating a particle size greater than 3000 mμ., whereas copper sulphide sols tested with Gradocol membranes kindly supplied by Prof. C. L. Oakley contained inhibitory particles lying between 230 and 330 mμ. Differences in behaviour between the two colloids therefore might reflect this large difference in particle size. It is possible that if finer sulphur suspensions could have been produced these would have behaved more like the copper sulphide sols in experiments on annulment of the inhibitions. The work of Libenson *et al.* (1953) suggests that these finer suspensions of sulphur would also have been more inhibitory. The difficulty of producing colloidal sulphur solutions of any particular particle size prevented this idea being tested.

Inhibition of micro-organisms by colloidal copper sulphide

A number of workers have studied the action of colloidal sulphur on micro-organisms (Lawson, 1934; Massey & Snyder, 1936; Weld & Gunther, 1947; Libenson *et al.* 1953), and in general they found it more inhibitory for Gram-positive than Gram-negative organisms. Inhibitory casein hydrolysate medium was prepared by adding 10 mg. Cu/l. and autoclaving. Its effect on the growth of a number of common organisms was examined, unheated medium being used as a control. All strains of staphylococci tested (10) were inhibited, as were representative strains of *Bacillus subtilis*, *B. polymyxa*, *B. cereus*, and *B. pumilus*. *Brucella abortus* and *Br. melitensis* were inhibited whilst *Br. bronchisepticus* was unaffected. *Vibrio comma*, *Pseudomonas aeruginosa*, *Aerobacter aerogenes*, *Escherichia coli*, *Salmonella typhi*, *Shigella paradysenteriae*, *Proteus vulgaris* and *Pr. mirabilis* were not inhibited. In general copper sulphide behaved like sulphur in being more active against Gram-positive than Gram-negative organisms.

The inhibition of bacterial growth by sulphides of heavy metals other than copper

Although O'Meara & Macsween (1936, 1937) showed that copper was responsible for the heat-induced inhibitory action of peptones, there appeared no reason why other heavy metals whose sulphides were insoluble should not also be inhibitory. Few heavy metal sulphides are as insoluble as copper sulphide, and their formation in autoclaved medium therefore was unlikely since insufficient hydrogen sulphide would be produced for their solubility products to be exceeded. Hydrogen sulphide formed during growth of an organism might, however, lead to sulphide formation and the effects of these sulphides on staphylococci was therefore investigated. The metals chosen for examination were iron, manganese, nickel, cobalt, zinc, cadmium, mercury, lead, silver and gold. Many of these metals are present in peptone, usually in traces but sometimes in considerable amounts. Silver and gold, though not likely to be present in media, were of interest because of their close relationship with copper in the Periodic Table. Neither of these metals could be added as salts to liquid medium and autoclaved, since the metal ion was toxic for CN 59. Their sulphide sols, however, were readily prepared by the technique used for preparing copper sulphide. These sols were a clear golden brown and were more stable than colloidal copper sulphide in that they could be boiled without change of colour. When added to casein hydrolysate medium they inhibited the test organism but had only about one-tenth the activity of copper sulphide. Attempts to produce stable sulphide sols of other heavy metals were unsuccessful; they flocculated when they were added to, or were incubated with, the culture medium, so there was little prospect of settling the question of the inhibitory action of these sulphides until some method for stabilizing them was devised.

It was found that the inhibitory effect of copper sulphide could be demonstrated by placing a disk of filter-paper impregnated with sodium sulphide on the surface of a poured plate of nutrient agar inoculated with CN 59 and containing copper chloride. Other heavy metals were tested by this technique as it offered a solution of the problem of stabilization. The metal was incorporated in the nutrient agar usually as the chloride or sulphate at a level of 10 mg./l. Sodium sulphide solutions with concentrations between 1 and 2·5 % (w/v) were used for impregnating the disks and CN 59 was the test organism. Co, Ni, Pb, and Zn showed no inhibition either in the medium itself or round the sodium sulphide disk but Cd gave slight inhibition round the disk. Copper gave a zone of complete inhibition with clear-cut edges, together with a larger shallow zone of decreased growth (Pl. 1, fig. 2). The second zone, which does not show well in the photograph, was thought to be due to hydrogen sulphide which would diffuse through the plate faster than the sodium sulphide from which it was formed by hydrolysis. Mercury completely inhibited growth in the plate, but heavy growth was obtained round the sodium sulphide disk. Iron and manganese both gave sharply defined zones of inhibition round the disk (Pl. 1, figs. 3, 4) but the zones were not as big as with copper nor was any secondary zone seen. Disks impregnated with sodium sulphide on plates of nutrient agar were used as controls, but in no case was inhibition observed. Indicator solution was flooded over plates which showed inhibitory zones to see whether large pH changes had occurred because of interaction of metal salt and sodium sulphide or hydrolysis of sodium sulphide; no changes were detected and the zones therefore appear to be due to sulphide formation. Copper produced by far the most inhibitory sulphide but the fact that iron and manganese sulphide could inhibit the growth of staphylococci though relatively weakly was of considerable interest; the possible significance of this finding is discussed later.

DISCUSSION

How colloidal copper sulphide exerts its inhibitory action is not known. It is an extremely insoluble substance having a solubility product of $8{\cdot}5 \times 10^{-44}$ (Ephraim, 1943) and is not very reactive. It is therefore unlikely to combine with media constituents and render them unavailable to the organism. Being a colloid it cannot penetrate into the cell, but it might be adsorbed on the cell wall and there interfere with the ingress of essential metabolites or with enzymic transformations necessary for growth. The reversal of the inhibition by β-mercaptopropionic acid but not by cysteine or cystine suggests that specific enzyme systems are being interfered with, possibly those concerned with the utilization of sulphydryl groups which Fildes & Richardson (1937) showed were essential for the growth of staphylococci. Silver and gold sulphides are considerably less active than copper sulphide and as all these metals are closely allied in the Periodic System this may indicate a steric factor, the molecular dimensions of copper sulphide being such that it fits into an enzyme system in place of the usual substrate more readily than either gold or silver sulphides.

It might seem that colloidal sulphur is acting on the cocci in a different way from colloidal copper sulphide as its action is so easily reversed with cystine, cysteine, and mercaptoacetic acid, whereas copper sulphide inhibition is unaffected by these compounds. However, Schuhardt *et al.* (1952) showed with brucellas that annulment of sulphur inhibition could in many cases be explained by the chemical reaction of sulphur with the annulling agent or with media constituents. The difference in behaviour of the two colloids may therefore be a reflexion of differences in chemical reactivity rather than a fundamental difference in mode of action. The problem is complicated by the fact that it is impossible to be certain that apparent annulment of inhibition is not due to flocculation of the inhibitory colloid. It is much more difficult to prepare stable colloidal sulphur suspensions than copper sulphide sols, and it is noticeable that when the particle size of the copper sulphide sol is increased by heating, the resultant sols are much less stable and flocculate readily. This flocculation is easily detected, since copper sulphide is coloured. It is not easy to detect the flocculation of dilute sulphur suspensions, particularly in the presence of bacteria.

Because of the work of O'Meara & Macsween (1936, 1937) copper has largely been eliminated from commercial peptones and precautions are normally taken to prevent its entering culture media during preparation. The lack of toxicity of most common heavy metal sulphides, coupled with the fact that those with an inhibitory action have a larger solubility product than copper sulphide, means that the sulphides of metals other than copper do not present a problem in media making. A number of bacteria produce sufficient hydrogen sulphide during growth for the solubility product of the sulphides of these metals then to be exceeded. The darkening of media during the growth of many anaerobes, for instance, is commonly attributed to the production of colloidal iron sulphide. The demonstration in the present work of the biological activity of iron sulphide may therefore have some importance. It seems unlikely that iron sulphide would affect the growth of these organisms, since heavy growth would be necessary to produce sufficient hydrogen sulphide for its formation, but it might well affect enzyme systems which produce special metabolic products, such as bacterial toxins, or might even interact with such products and decrease or abolish their activity. Cooked meat particles are often added to anaerobic media to ensure good yields of toxins. Besides providing a nidus for initiating growth (Knight, 1941) and introducing reducing systems to assist in the production of anaerobic conditions (Lepper & Martin, 1929), the meat particles might also help toxin production by absorbing inhibitory colloidal sulphides. If this were so, even better results would be expected if these colloids could be excluded from contact with the growing bacteria. From this point of view the experiments of Sterne & Wentzel (1950) with *Clostridium botulinum* are of interest. They grew this organism in cellophan sacs, the culture medium being on the outside and the organism inoculated into saline inside the sac. Toxins 50 to 100 times as potent as those prepared in the conventional way were obtained. *Cl. botulinum* is a strong hydrogen sulphide producer and this gas would pass rapidly through the membrane into the surrounding medium where it could react with iron and other heavy metals. The sulphides formed would be prevented by the cellophan membrane from re-entering the saline containing the growing organisms and would not therefore be able to affect either the growth or toxin-producing capacity of the organisms.

Although it is not known why copper-containing media fail to develop inhibition when autoclaved in screw-capped bottles or sealed ampoules it is possible to suggest a mechanism for this failure. If the decomposition of cystine on heating is a reversible reaction, an equilibrium will be attained when the heating is carried out in a closed system such as a sealed ampoule. Under any given conditions of temperature and pressure a limited amount of hydrogen sulphide would therefore be produced, and if this were insufficient with the copper present to exceed the solubility product of copper sulphide, the resulting solution would not be inhibitory. However, if hydrogen sulphide could escape, as would be the case with cotton-wool plugged containers, the equilibrium would be upset and the breakdown of cystine would proceed throughout the heating period. Much of the extra hydrogen sulphide produced would pass through the plug but more would also be available for reaction with any copper present and the likelihood of the solubility product of copper sulphide being exceeded would be much increased.

I wish to thank Miss Ann Norton for her invaluable technical assistance throughout this work.

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EXPLANATION OF PLATE

Growth of *Staphylococcus aureus* round disks impregnated with sodium sulphide solution (2·5 %) on nutrient agar pour plates, with or without added salts.

Fig. 1. Nutrient agar.
Fig. 2. Nutrient agar with 10 mg. Cu''/l.
Fig. 3. Nutrient agar with 10 mg. Fe'''/l.
Fig. 4. Nutrient agar with 10 mg. Mn''/l.
Fig. 5. Nutrient agar with 10 mg. Ni/l.$''$
Fig. 6. Nutrient agar with 10 mg. Hg''/l.

(*Received* 4 *January* 1954)

Journal of General Microbiology, Vol. 10, *No.* 3

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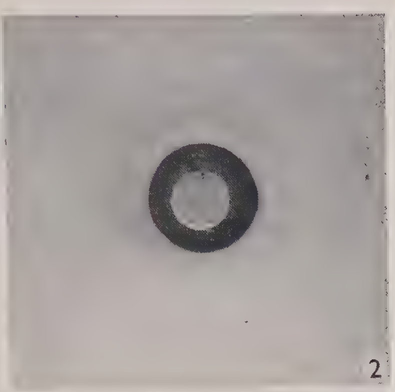
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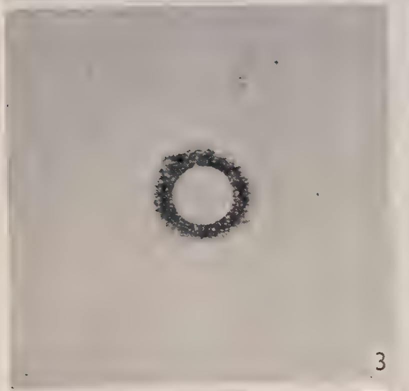
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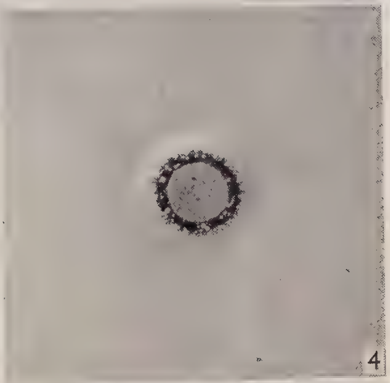
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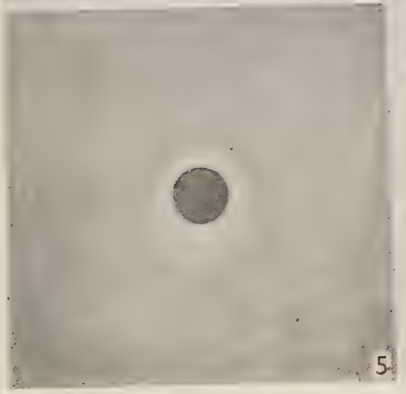
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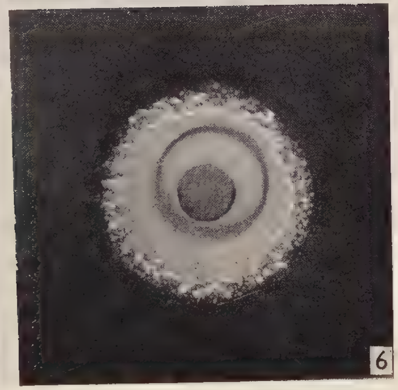
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A. J. WOIWOD—INHIBITION BY METAL SULPHIDES AND SULPHUR. PLATE 1

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Effects of some Purine analogues on Tobacco Mosaic Virus

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SUMMARY: When sprayed on the leaves of tobacco plants inoculated with tobacco mosaic virus 8-azaguanine caused a delay in the production of virus, and a delay in the development of systemic infection. From alkaline hydrolysates of the nucleic acid prepared from virus isolated from 8-azaguanine-treated plants, a compound was isolated which had the expected properties of 8-azaguanylic acid. This evidence together with analytical data showed that in tobacco mosaic virus from 14-day-old infections in 8-azaguanine-treated plants, about 3–4% of the guanine in the virus nucleic acid was replaced by 8-azaguanine. This incorporation of 8-azaguanine appeared to reduce the infectivity of the virus. Several other purine analogues were ineffective against tobacco mosaic virus. A serological-chromatographic method is described for the estimation of small amounts of tobacco mosaic virus in crude plant extracts.

The guanine analogue, 8-azaguanine, has a marked inhibitory effect on lucerne mosaic and cucumber mosaic viruses (Matthews, 1953*a*). The compound has much less effect on disease development with tobacco mosaic virus (TMV). However it is possible with this virus to make a more detailed investigation of the inhibition. The work described here shows that 8-azaguanine is incorporated into TMV nucleic acid, and suggests that the compound acts as an inhibitor because virus particles containing it are incapable of further multiplication. A number of compounds related to 8-azaguanine were ineffective. A preliminary report of some of these results has already appeared (Matthews, 1953*b*).

MATERIALS AND METHODS

Methods of growing plants, carrying out virus inoculations and treatments with compounds, and testing compounds for effects on disease development were as described by Matthews (1953*a*). The virus strains used were: the 'Type', 'Astoria', 'Yellow' and 'Tobacco Leaf Curl' cultures maintained at the A.R.C. Plant Virus Research Unit, and a New Zealand 'type' culture which produced similar symptoms to the Cambridge type strain but was not necessarily identical with it.

Isolation of virus. Harvested leaves were frozen, minced, and allowed to thaw slowly at room temperature, and the sap was expressed through muslin. The sap was clarified by heating to 55° for 3–5 min. followed by centrifugation at 2200 r.p.m. for 10 min. Virus was precipitated from the supernatant fluid by addition of one-half volume of saturated ammonium sulphate. After centrifugation, the virus-containing pellet was taken up in water, dialysed, centrifuged to remove insoluble impurities and precipitated a second time with ammonium sulphate followed by dialysis. Yields of virus were estimated by dry weights, by micro-Kjeldahl nitrogen determinations, or by the density of the solutions in ultraviolet light, assuming that 1 mg. virus/ml. gives a density of 3·2 at 260 mμ. in a 1 cm. cell.

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* Present address.

Isolation of nucleic acid. Nucleic acid was isolated from the purified virus preparations by heating to about 95° (Bawden & Pirie, 1937) in neutral solution in the presence of small amounts of salt, centrifuging to remove the protein thus coagulated and precipitating the nucleic acid with two volumes of ethanol. The precipitate was then redissolved in slightly alkaline solution, centrifuged at 8000 r.p.m. to remove further traces of protein, and reprecipitated with ethanol.

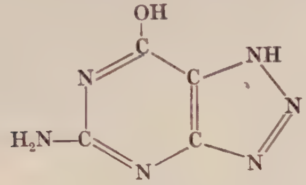
Analytical procedures. The chromatographic and electrophoretic procedures used were those described by Markham & Smith (1949, 1950*a*, *b*, 1952*a*). Nitrogen was estimated by the micro-Kjeldahl method of Markham (1942) and phosphorus by the method of Allen (1940).

The solvent systems used for paper chromatography were: *iso*propanol + HCl (*iso*propanol, 170 ml.; conc. HCl, 44 ml.; water to 250 ml.); *iso*propanol + NH_3 (70 % *iso*propanol in water (v/v) with NH_3 in the vapour phase); ammonium sulphate + water (sat. $(NH_4)_2SO_4$, 80 vol.; *iso*propanol, 2 vol.; 0·5 M-sodium acetate, 18 vol.).

Fluorescence printing. The method of printing chromatograms and paper electrophoresis strips by the fluorescence of the spots in ultraviolet light, which was briefly described by Markham & Smith (1950*a*), proved particularly useful. In this method, a thin film of cellulose nitrate ('Celluloid', The Autotype Co. Ltd., Brownlow Road, London, W. 13) which is opaque to ultraviolet light, is placed between the filter-paper and the photographic paper. Cellulose or cellulose acetate film is ineffective, being transparent to ultraviolet light. Thus only fluorescent light from the spots or from the paper will affect the photographic paper. The limitation in the sensitivity of the method is the background fluorescence of the filter-paper. Using the lamp and filter described by Markham & Smith (1951), cellulose nitrate film 0·08 mm. thick, and Kodak grade 0 or 1 bromide paper, exposures of 10–20 sec. are required for Whatman's no. 1, and 30–50 sec. for Whatman's no. 3 filter-paper. It was found that 8-azaguanine fluoresces strongly under both acid and alkaline conditions, while guanine compounds fluoresce strongly, if at all, only under acid conditions; for 8-azaguanine the method is about 10 times as sensitive as detecting the spots by absorption. In the absence of relatively large amounts of ultraviolet light absorbing material it is possible to detect about 0·3 μg. of 8-azaguanine.

Serological-chromatographic method for estimation of virus. The following method which takes advantage of the sensitivity and specificity of both the precipitin reaction and chromatographic procedures was used to estimate TMV in small samples of leaf sap. Frozen leaf samples are ground in a small volume of water, the suspension heated to 55° for 5 min. and the green precipitate removed by centrifugation. The supernatant liquid is mixed with a small excess (previously determined) of an anti-tobacco mosaic virus serum. The mixture is incubated for 1 hr. at 50° and overnight at room temperature. The specific precipitate is centrifuged down, washed once with 0·14 M-NaCl and carefully dried. The precipitate is digested overnight in a known small volume of 2 N-KOH, and neutralized with the appropriate amount of $HClO_4$. The $KClO_4$ precipitate is centrifuged off. Portions of the supernatant fluid are then subjected to chromatography in the *iso*propanol + ammonia solvent. The guanylic acid spot and the slightly faster moving spot containing the other three mononucleotides are then eluted in 0·1 N-HCl and estimated spectrophotometrically. The method is useful for amounts of virus in the range 0·2–10·0 mg. giving recoveries around 90 %.

Compounds. Guanosine (Light and Co.); 8-azaguanine (I), 8-azahypoxanthine (II), 8-azaisoguanine (III), 8-azaxanthine (IV), dimethylamino-8-azaguanine (V) (given by American Cyanamid Co.), 5-amino-7-hydroxy-1, 2, 4, 6-tetraazaindene (VI) (given by Imperial Chemical Industries).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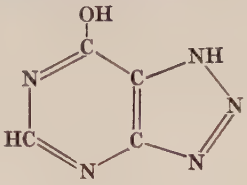
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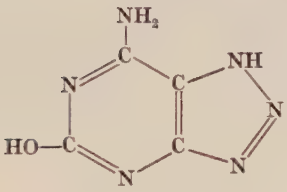
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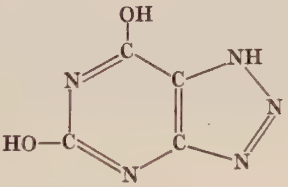
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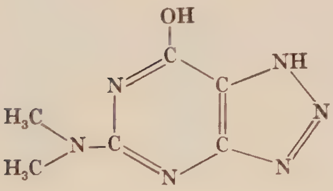
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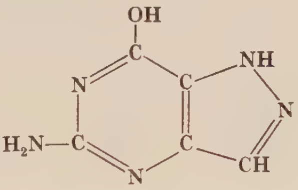
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RESULTS

Effect of 8-*azaguanine on virus development*

When sprayed on the leaves of young tobacco plants at concentrations of *c.* 0·01 M in solution in sodium bicarbonate, 8-azaguanine caused a delay of several days (usually 3–6) in the appearance of systemic symptoms of TMV infection but never prevented eventual systemic development. The compound had this effect on all the strains of the virus that were tested, namely: 'Type' (Cambridge and New Zealand isolates), 'Yellow', 'Tobacco Leaf Curl' and 'Astoria'. Inoculation tests for the presence of virus in uninoculated young leaves showed that this delay in the appearance of symptoms reflected a delay in the movement of virus from inoculated leaves. In inoculated leaves, 8-azaguanine caused a delay in the production of virus measured by infectivity, by yield of virus extractable by the ammonium sulphate procedure, or by particle counts using electron microscopy (Williamson & Matthews, unpublished). Fig. 1 illustrates this effect when the amount of virus was estimated by infectivity.

Incorporation of 8-*azaguanine into the virus nucleic acid*

To prepare virus for analytical work young tobacco plants were inoculated with a type strain in highly infective expressed tobacco sap. The day after inoculation and subsequently every 2 or 3 days 'treatment' plants were sprayed with 0·01 M-8-azaguanine solution. Plants were usually harvested about 14 days after inoculation and the virus was isolated by the ammonium sulphate procedure. The first evidence that incorporation of 8-azaguanine was taking place came from base analyses on the nucleic acid from virus from treated and untreated plants. The nucleic acid samples were hydrolysed for 1 hr. at 100° in N-HCl and chromatographed in *iso*propanol + HCl, a solvent system in which 8-azaguanine runs with adenine. The results of a number of analyses summarized in Table 1, indicated a deficiency of about 3·5% in guanine in the nucleic acid from virus from treated plants. This deficiency in guanine was accounted for (within the limits of experimental error) by a corresponding increase in adenine. This result would be expected if 8-azaguanine was replacing some of the guanine in the virus from treated plants. When acid-hydrolysed nucleic acid from virus from treated plants was run on two-dimensional chromatograms (first solvent: *iso*propanol + HCl; second solvent: *iso*propanol + NH_3) a small fluorescent spot was observed behind adenine in the second solvent system, in the position expected for 8-azaguanine. The fluorescent material isolated by this procedure had an electrophoretic mobility on filter-paper in 0·05 M-borate buffer at pH 9 corresponding to that of 8-azaguanine.

Table 1. *Molar ratios of bases in nucleic acid of tobacco mosaic virus from control and 8-azaguanine-treated plants*

	Molar ratios (means of seven determinations)			
Base	Control	8-Azaguanine treated	Difference, control − treated	Difference required for significance at 0·01 *P*
Guanine	0·979	0·943	−0·036	0·024
Adenine	1·103	1·134	+0·031	0·031
Cytosine	0·817	0·815	−0·002	0·021
Uracil	1·101	1·110	+0·009	0·058

However, isolation of a compound as the base is not proof of incorporation into the nucleic acid, and previous claims for incorporation of 8-azaguanine into nucleic acids (Mitchell, Skipper & Bennett, 1950; Henrich, Dewey, Parks & Kidder, 1952) are open to this criticism. In alkaline hydrolysates of virus nucleic acid, where all the bases that were in polynucleotides should appear as nucleotides, we have sometimes detected small amounts of free 8-azaguanine. This material was presumably a contaminant surviving both the virus purification and nucleic acid isolation procedures. Therefore to demonstrate incorporation of a compound it would appear necessary to isolate the material as a nucleotide or at least as the nucleoside.

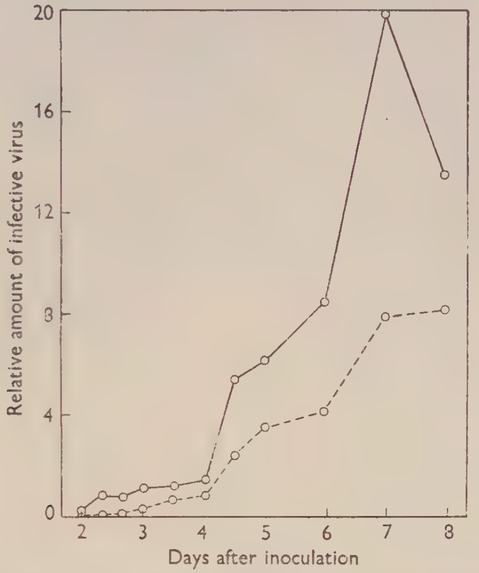

Fig. 1. Effect of 8-azaguanine on tobacco mosaic virus in inoculated tobacco leaves. Solid line: control, untreated plants. Dotted line: 8-azaguanine-treated plants. Test samples were compared with a standard preparation by half leaf comparisons on *Nicotiana glutinosa*. Relative amount of infective virus is the ratio:

$$\frac{\text{no. of local lesions on test half leaves}}{\text{no. of local lesions on standard half leaves}}.$$

In attempting to isolate 8-azaguanylic from alkaline hydrolysates of nucleic acid advantage was taken of the fact (determined approximately by paper electrophoresis) that the pK's of the hydroxyl and amino groups in the triazolopyrimidines are lower than those for the corresponding purines. In the case of 8-azaguanine the pK of the hydroxyl group is 2–3 pH units lower than that of guanine.

When nucleic acid from virus from 8-azaguanine-treated plants was hydrolysed with N-sodium hydroxide (at room temperature for 24 hr.) and subjected to electrophoresis on filter-paper in borate buffer at pH 9, a fluorescent band was found, running in a positive direction slightly ahead of uridylic acid, the fastest moving of the naturally occurring mononucleotides at this pH.

This band, which was absent from control preparations, moved about 1·4 times as fast as 8-azaguanine, and had approximately the mobility to be expected for 8-azaguanylic acid. This procedure was used to isolate the fluorescent material which was further purified by chromatography in *iso*propanol + NH_3. Table 2 gives the approximate R_F values of the fluorescent material and other compounds in two solvent systems.

Table 2. *Approximate R_F values in two solvent systems*

	Solvent systems	
	*Iso*propanol + ammonia	Ammonium sulphate + water
	R_F values	
Compound		
Guanine	0·33	0·16
Guanylic acid	0·08	*b* 0·33 *a* 0·42
8-Azaguanine	0·27	0·17
8-Azaguanylic acid	0·05	*b* 0·33 *a* 0·40

In the ammonium sulphate + water solvent, the fluorescent material divided into two spots of roughly equal size running in the expected positions for 8-azaguanylic acids '*a*' and '*b*'. This is strong evidence for the production of 8-azaguanylic acid from polynucleotides through the 'cyclic' 2′ : 3′-monohydrogen phosphates (Markham & Smith, 1952*b*) since the '*a*' and '*b*' isomers are stable to alkali.

The ultraviolet absorption spectrum of the fluorescent material isolated from the filter-paper before and after acid hydrolysis is shown in Fig. 2. Guanylic acid has an absorption maximum near 255 mμ., while with guanine the maximum is near 247 mμ. The fluorescent material had a maximum at 255 mμ., which shifted on acid hydrolysis to about 247 mμ., the position of maximum absorption for 8-azaguanine. After acid hydrolysis the material had the chromatographic and electrophoretic properties of 8-azaguanine. The material contained phosphorus, and the base : phosphorus ratio from two estimations (made on 0·5–1·0 μg. of phosphorus) was 1·0 : 0·83. From the above facts we conclude that the compound is 8-azaguanylic acid (as a mixture of 8-azaguanosine 2′ and 3′ phosphates) and therefore that 8-azaguanine had been incorporated into the nucleic acid of tobacco mosaic virus.

Effect of 8-azaguanine incorporation on infectivity

It is possible to compare the infectivity of two virus preparations by estimating the total amounts of virus material present by some physical or chemical means and comparing these estimates with those obtained by infectivity measurements on the same preparations. By such comparisons we attempted to determine whether virus preparations containing 8-azaguanine were less infectious than control preparations. In a number of experiments using material from 14-day-old infections, K. Williamson and the writer estimated the amount of virus by infectivity, by extraction of virus and estimation of yield by nitrogen, and by particle counts using electron microscopy. In no case did the infectivity per particle or per weight of virus extracted, in virus from treated plants, differ significantly from control virus preparations. However, material from 3-day infections from treated plants consistently gave a lower ratio of infectivity to total virus than did control preparations, when total virus was measured by the amount of virus isolated by the ammonium sulphate precipitation procedure, by precipitin end-point, or by the serological-chromatographic method. In these experiments young tobacco plants were sprayed with 0·01 M-8-azaguanine on the 2 days following inoculation, and inoculated leaves were harvested on the third day. Control plants were sprayed once with 0·02 M-8-azaguanine about 2 hr. before harvesting. The relative infectivities of control and treated expressed sap were determined by inoculation to half leaves of *Nicotiana glutinosa*. Appropriate dilutions were determined by preliminary tests, and were adjusted to give an average of between 10 and 100 local lesions/half leaf, and approximately to equalize the numbers given by both samples. The data, summarized in Table 3, suggest that roughly half the virus particles in 8-azaguanine-treated plants were rendered non-infectious.

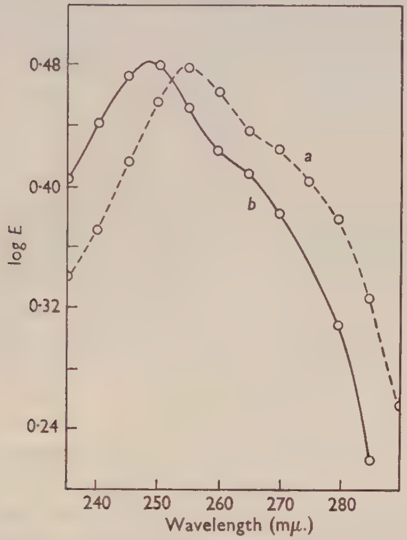

Fig. 2. Ultraviolet absorption spectrum in N/10-HCl, of 8-azaguanylic acid from tobacco mosaic virus nucleic acid: (*a*) before hydrolysis; (*b*) after hydrolysis in N-HCl for 1 hr. at 100°.

Distribution of 8-azaguanine among the virus particles

Using the size and density of the fluorescent spot of 8-azaguanylic acid obtained from a known total density of nucleotides as an approximate estimate of amount, it was found that, in 14-day infections, there was much more 8-azaguanine in virus from inoculated leaves than in that from systemically

Table 3. *Effect of 8-azaguanine incorporation on the infectivity of tobacco mosaic virus in 3-day-old infections*

Expt. no.	Ratio of amount of virus in tobacco sap 8-azaguanine-treated/control determined by				Ratios		
	(*a*) Infectivity	(*b*) Extraction of virus	(*c*) Serological chromato-graphic method	(*d*) Precipitin end-point	*a*/*b*	*a*/*c*	*a*/*d*
1	0·42	0·93	—	—	0·45	—	—
2	0·20	0·37	—	—	0·54	—	—
3	0·29	0·82	—	—	0·35	—	-
4	0·25	—	0·50	0·50	—	0·50	0·50
5	0·35	—	0·61	0·71	—	0·57	0·49
6	0·40	—	0·65	0·71	—	0·62	0·55

infected leaves. This suggests that early-formed virus contains more 8-azaguanine than that formed later, and therefore that in the preparations used for analyses the 8-azaguanine was not randomly distributed among the virus particles.

Attempts to fractionate 8-azaguanine containing virus preparations

By centrifugation. TMV was prepared from 8-azaguanine-treated plants 14 days after inoculation. The method used was that described by Bawden & Pirie (1945) which minimizes aggregation of the virus rods during extraction. The extracted sap was centrifuged at 16,000 r.p.m. in a Servall S.S. 2 centrifuge for 1 hr. Electron microscopic examination showed that the pellet consisted largely of long rods, while in the supernatant liquid short rods predominated. Nucleic acid was prepared from both samples. There appeared to be no difference in the amount of 8-azaguanylic acid as estimated by the size and intensity of the fluorescent spot.

By serological cross-absorptions. It seemed possible that incorporation of 8-azaguanine into the virus nucleic acid might cause some change in the protein part of the virus which could be detected by serological means. Two antisera were prepared in rabbits against control virus, and 2 against 8-azaguanine containing virus. Cross-absorption tests between antisera against control and 8-azaguanine containing virus failed to reveal any differences.

By milling plant fibre. Bawden & Pirie (1945) showed that the fibrous leaf residue remaining after the sap had been expressed yielded further virus material when processed in a colloid mill. With 8-azaguanine-treated plants, virus obtained by milling of the fibre contained about the same amount of 8-azaguanylic acid as that obtained from the sap.

Effect of reversal of 8-azaguanine inhibition by guanosine

With lucerne mosaic virus the inhibitory activity of 8-azaguanine is annulled by several normal purines (Matthews, 1958*a*). To test the effect of a normal purine on 8-azaguanine incorporation, guanosine was used rather than guanine because of its much greater solubility. Four sets of tobacco plants were inoculated with type TMV and 2 hr. later each set was given one of the treatments listedb elow. Treatments were repeated on the two following days. Plants were harvested on the fifth day and the virus extracted and estimated. Yields (expressed as mg./ml. in clarified sap) were:

Control, water sprayed	1·7
Sprayed 8-azaguanine 0·005 M	0·96
Sprayed guanosine 0·01 M	2·1
Sprayed 8-azaguanine 0·005 M + guanosine 0·01 M	1·9

The size of the 8-azaguanylic acid spot obtained from similar amounts of alkaline-hydrolysed nucleic acid showed that in the virus from plants treated with guanosine plus 8-azaguanine, there was very much less incorporation than in that from plants treated with 8-azaguanine alone.

Effect of other purine analogues

8-Azahypoxanthine, 8-azaxanthine, 8-azaisoguanine and dimethylamino-8-azaguanine had no effect on disease development or on yield of virus, and no 8-azaguanylic acid could be detected in virus preparations from treated plants. We have so far had insufficient 8-azaadenine for adequate tests. The compound 5-amino-7-hydroxy-1, 2, 4, 6-tetraazaindene had no effect on disease development.

DISCUSSION

The isolation of a compound with the expected properties of 8-azaguanylic acid, together with the analytical data on the ratios of bases in the nucleic acids, demonstrates that in TMV from 14-day infections in plants sprayed with 8-azaguanine, about 3 % of the guanine residues are replaced by 8-azaguanine. The problem of estimating the specific activity of virus preparations is a difficult one, and none of the methods available for estimating TMV is entirely free from criticism. However, all the methods used in this work agree in suggesting that in 3-day infections treated with 8-azaguanine roughly half the virus particles are rendered non-infectious. There are two possible alternative explanations for this result. First, the 8-azaguanine preparations might contain a contaminant not present in the control preparations. Such a contaminant would have to contain nucleic acid and precipitate with tobacco mosaic virus antiserum. It would also need to be stable to slow thawing of the plant extract, heating to 55° for 3–5 min. and precipitation with one-third saturated ammonium sulphate. As Pirie (1950) points out, such stability is unlikely for a plant nucleoprotein. Secondly, normal immature non-infectious forms of the virus might exist and be present to a greater extent in the 8-azaguanine containing preparations since less multiplication had taken place. There is at present no evidence for the existence of such forms of the virus. To explain the present results, the immature form should have the properties noted above for a contaminant. Bawden & Pirie (1945) separated tobacco mosaic virus preparations into fractions of different average sizes. Fractions containing a predominance of small particles were less infectious. However, they found no consistent variation in relative amount of the less infectious fraction, in preparations harvested during the period of virus increase from 10 days to 4 weeks after inoculation. There was thus no evidence that the less infectious fractions were immature forms. Since there is evidence that the 8-azaguanine is not randomly distributed among the virus particles in a preparation, the early-formed virus containing more than that formed later, we can make no estimate of how many guanine residues must be replaced by 8-azaguanine to make a particle non-infectious. Indeed the effectiveness of the compound may depend on particular positions being occupied rather than on the total number of guanine residues replaced.

Treatment of plants with 8-azaguanine delays the production of virus when amount of virus is measured by infectivity or by other methods. We have calculated from counts made on thin sections of tobacco leaf tissue that not more than about 10 % and more probably about 0·1 % of the cells of a tobacco leaf are infected when inoculated with a highly infective virus preparation. The rate of increase of virus will therefore depend on two effects: rate of multiplication in individual cells and rate of invasion of fresh cells. For this reason alone, little can be inferred from growth curves, for plant viruses at least, regarding the effect of a compound on actual rate of virus multiplication.

The evidence so far obtained suggests that 8-azaguanine is sufficiently similar to guanine to be incorporated into the virus nucleic acid, but different enough to make the nucleic acid containing it non-functional, thus rendering the virus particle incapable of further multiplication. The compound appears to be most effective at the site of inoculation, where its concentration is highest and the amount of virus multiplying is lowest. Most of the 8-azaguanine absorbed is fairly rapidly inactivated by conversion to 8-azaxanthine (Matthews, 1953*b*).

It seems unlikely that 8-azaguanine itself will be of any practical value in control, at least for the plant viruses we have tested. However, modifications of the compound which resulted in greater incorporation of 8-azaguanine might give greater inhibition. A compound with any of the following properties might be incorporated to a greater extent than 8-azaguanine: (*a*) less susceptibility to deamination *in vivo*; (*b*) greater solubility allowing greater absorption; (*c*) more directly on the path of incorporation into nucleic acids.

Compounds which may have some of these properties are:

(*a*) *Compounds containing 8-azaguanine*: (i) 8-azaguanosine; (ii) 8-azaguanosine diphosphates (2′ : 5′-, 3′ : 5′-, or 2′ : 3′ cyclic 5′-); (iii) 8-azaguanylic acids (2′-, 3′-, 5′-, or 2′ : 3′ cyclic phosphates); (iv) dinucleotides, etc., containing 8-azaguanine.

(*b*) *Potential precursors of 8-azaguanine*: (i) 5′ amino-1 H, 1, 2, 3, triazole-4-carboxyamide and its riboside and ribonucleotides; (ii) 8-azapurine substituted in the 2′ position.

There is at present no evidence that units larger than mononucleotides can be incorporated into nucleic acids. If, however, it should be found that small polynucleotides can be utilized in nucleic acid synthesis, it would be of interest to determine the relative inhibitory effects of 8-azaguanine incorporated into small polynucleotides of various types containing the normal bases. It is possible that such compounds could control, to some extent, the distribution of the 8-azaguanine residues in the whole nucleic acid. They might also lead to a preferential incorporation into one type of nucleic acid and thus allow the difference in sensitivity to the compound between host tissues and virus to be increased.

In work to be published elsewhere we have confirmed the finding of Commoner & Mercer (1952) that 2-thiouracil inhibits the development of tobacco mosaic virus. Jeener & Roseels (1953) using ^{32}S labelled 2-thiouracil claimed on the basis of radioactivity data that under conditions giving about 50% virus inhibition thiouracil replaced 20% of the uracil in TMV nucleic acid. We have so far been unable to confirm this observation using methods that should have been sensitive enough to detect the presence of thiouracil if only 5% of the uracil in the nucleic acid had been replaced by this compound.

In searching for other purine or pyrimidine-like compounds which could be incorporated into nucleic acids in may be more profitable to examine those with modifications in the purine or pyrimidine ring, rather than in the substituent groups, although the facts are as yet too few to make any firm generalization possible. It may be that for some reason 8-azaguanine is unique in being able to be incorporated into nucleic acids. If, however, other types of analogue can be found which will replace normal bases, we may obtain information about the structure of nucleic acids in relation to their function of a type not possible with either degradative or X-ray studies. For example the data obtained with 8-azaguanine suggest that in tobacco mosaic virus nucleic acid the carbon of position 8′ in guanine is not essential for guanine incorporation and that a nitrogen in position 8′ can replace carbon during incorporation but is unsuitable in the process of reduplication. The facts that dimethylamino-8-azaguanine had no effect on virus development and could not be shown to be incorporated suggest that the integrity of the amino group is essential for incorporation. The modification of guanine in which the carbon of position 8 and the nitrogen of position 9 are interchanged was ineffective against several plant viruses. This is probably because a nitrogen atom is necessary in position 9 for the formation of a nucleotide of the normal configuration.

I wish to thank Dr R. Markham for much useful discussion; Mr P. R. Fry for a number of virus preparations, and the American Cyanamid Co. and Imperial Chemical Industries for supplies of compounds.

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(*Received* 6 *January* 1954)

MITCHELL, P. & MOYLE, J. (1954). *J. gen. Microbiol.* **10**, 533–540.

The Gram Reaction and Cell Composition: Nucleic Acids and other Phosphate fractions

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SUMMARY: The content of ribonucleic acid (RNA), excess organic phosphate (XSP) in the RNA fraction, deoxyribonucleic acid (DNA), acid-soluble inorganic phosphate (AI) and organic phosphate (AO), and the lipid phosphate (LP) and lipid weight (L) have been determined in some ten Gram-positive and ten Gram-negative organisms, grown in the same media and harvested at the same phase of growth. A statistical analysis of the results shows that the RNA and DNA contents of the Gram-positive and Gram-negative groups do not differ significantly; the percentage of lipid phosphorus is only half as great in the Gram-positive as in the Gram-negative group; and XSP is present in all the Gram-positive organisms, but absent from all the Gram-negative ones. We suggest that a lipid-polyglycerophosphate-protein component may be a common structural feature of the cell envelopes of all Gram-positive organisms. The mechanism of the Gram reaction is considered in the light of our observations.

The Gram staining reaction has generally been considered to be caused by a special cell component, and opinion has been more or less equally divided as to whether this component is a substance which adsorbs the dye-iodine complex specifically or whether it is a barrier which prevents the outward diffusion of this complex. In either case, it would be expected that the capacity for retaining the dye-iodine complex would be reflected in the composition of the cells. Since Gram introduced his method of staining in 1884, it has become clear that the individual members of the Gram-positive and Gram-negative groups of organisms have in common not only their respective staining characteristics but also many general physiological characteristics. The study of Gram staining is therefore all the more interesting, since an explanation of the mechanism must involve a description of physiologically important differences of composition and structure between the two groups.

Very few comparative studies have been made on the chemical composition of Gram-positive and Gram-negative bacteria. For a small number of organisms, data are however available on the composition of the protein, the content of ribonucleic acid (RNA), the content of a phosphate ester (XSP) which occurs in the RNA fraction, and the magnesium content. The observations of Camien, Salle & Dunn (1945), Stokes & Gunness (1946) and Freeland & Gale (1947) on the amino acid composition of the whole protein of four Gram-negative and seven Gram-positive organisms showed no systematic differences except in the arginine content. In the Gram-positive organisms studied, the arginine represents *c.* 450 μmole/g. cell dry weight, whereas in the Gram-negative organisms it represents *c.* 900 μmole/g. A corresponding difference occurs in the composition of the protein of the cell walls of five

Gram-positive and two Gram-negative organisms. The cell walls of *Staphylococcus aureus* (*Micrococcus pyogenes*) (Mitchell & Moyle, 1951*c*) and of *Streptococcus pyogenes*, *Strep. faecalis*, *Micrococcus lysodeikticus* and *Sarcina lutea* (Salton, 1953) were found to contain practically no arginine, nor did they contain methionine, cysteine, phenylalanine or tyrosine. On the other hand, arginine and the sulphur-containing and aromatic amino acids were found in the cell walls of *Escherichia coli* and *Salmonella pullorum* (Salton, 1953). Stacey (1947) reported that whereas in several Gram-negative bacteria the ratio of ribonucleic acid to deoxyribonucleic acid (RNA/DNA) is only 1·3/1, in *Clostridium welchii* and streptococci it is greater than 8/1—implying that Gram-positive bacteria in general contain much more RNA than Gram-negative bacteria. Boivin (1948) and Mitchell & Moyle (1950) were unable to find the differences claimed by Stacey. But Mitchell & Moyle (1950, 1951*a*) observed that the RNA fraction (Schmidt & Thannhauser, 1945) of several Gram-positive bacteria contained a large excess of organic phosphate attributable to a new phosphoric ester (XSP) which was not present in Gram-negative organisms. In *Staph. aureus*, this ester was later shown to be a polyglycerophospho-compound which is a major component of the cell envelopes (Mitchell & Moyle, 1951*b*, *c*). We therefore found it difficult to accept the view that the essential component of the so-called Gram complex is magnesium ribonucleate (Henry & Stacey, 1943, 1946) and suggested that the new phosphoric ester might be part of the acidic cortical component which Gutstein (1925) considered to be responsible for the Gram staining reaction (Mitchell & Moyle, 1950). In response to our observations, Jones, Muggleton & Stacey (1950) reported that ribonucleic acids from different sources could substitute for one another in the 'Gram complex', and argued that '...one of the essential differences between *Cl. welchii* and the various Gram-negative organisms studied is the presence in the Gram-positive organism of a particular type of protein, which when linked to magnesium ribonucleate is able to retain the Gram stain'. But they gave no further information about the protein or about the mechanism of retention of the stain. Previously, however, Webb (1949) had demonstrated the important fact that the magnesium contents of Gram-positive and Gram-negative organisms differ significantly; the mean values for five Gram-positive and five Gram-negative bacteria grown on the same peptone medium were, respectively, *c.* 250 μmole Mg^{++} and *c.* 60 μmole Mg^{++}/g. cell dry weight.

The data presented in the present paper have been collected from work carried out in 1950 and 1951 on the XSP content, the RNA and DNA content, and the lipid phosphorus and acid-soluble inorganic and organic phosphorus contents of some ten Gram-positive and ten Gram-negative organisms, grown in the same media and harvested at the same phase of growth.

METHODS

Growth and preparation of organisms. The bacteria were grown in a casein digest medium containing 1% (w/v) glucose and 0·1% Marmite, except *Acetobacter pasteurianum* (NCIB 8088) which was grown in unhopped beer wort diluted with 4 vol. distilled water. The cultures of the aerobic organisms were aerated in rotated flasks (Mitchell, 1949) at 25°. The anaerobic organisms were grown in unstirred cultures in anaerobic jars at 25°. With one exception, the cells from measured volumes of the cultures were harvested by centrifuging during the phase of decelerated growth, washed twice with distilled water, freeze-dried and weighed. Cultures of *Neisseria catarrhalis* (NCTC 3622) were harvested at three different ages at which (i) practically all, (ii) about half, and (iii) practically none of the cells stained Gram-positive. The baker's yeast was the pressed Ark Yeast of Distillers Co. Ltd.; it was freeze-dried before use.

Fractionation of freeze-dried organisms. The methods of Mitchell & Moyle (1951*c*) and Mitchell (1953) were used to estimate the lipid weight (L), and the P content of the following fractions: the acid soluble inorganic phosphate (AI) and organic phosphate (AO); the lipid (LP); the RNA and XSP; and the DNA.

Gram staining. Heat-fixed films of the organisms were stained with 0·5% (w/v) aqueous crystal violet for 30 sec., mordanted with 1% (w/v) iodine in 2% aqueous KI for 30 sec. and differentiated with 95% ethanol. After washing with water, the films were counterstained with 0·1% (w/v) neutral red in 20 p.p.m. aqueous acetic acid for 2 min.

RESULTS

Table 1 describes the characteristics of the organisms: the cell-suspension density at harvesting in mg. cell dry weight/100 ml. culture (written mg. H/100 ml.); the lipid content in mg./g. cell dry weight (written mg. L/g.); the P content of the fractions in μmole P/g. cell dry weight; and the Gram reaction (written + or −). In some of the organisms, not all the fractions were estimated. The RNA fractions of all the organisms for which the RNA content is given were examined for inorganic phosphate corresponding to the phosphoprotein of Schmidt & Thannhauser (1945). In no case was there as much as 1 μmole inorganic P/g. cell dry weight.

The results for the Gram-positive and Gram-negative groups of bacteria (the Gram-variable Neisseria and the yeast being omitted) have been analysed separately and set out as histograms in Figs. 1–4. In each histogram, the arrow shows the arithmetic mean value for the fraction under consideration; the width of each block is equal to the standard deviation of the individual values from the mean; and the height of each block represents the number of organisms in which the value of the fraction falls within the range indicated by the width of the block.

There is no significant difference between the contents of DNA and RNA in the Gram-positive and Gram-negative groups of organisms (Fig. 1). There are, however, significant differences in the other phosphate fractions. The Gram-positive organisms contain more of the acid-soluble fractions AI and AO than the Gram-negative organisms (Fig. 2). The lipid weight (Fig. 4) does not differ significantly in the two groups, but the mean phosphorus content of the lipid (Figs. 3, 4) is 1·65% (w/w) in the Gram-positive group and 3·3% in the Gram-

Table 1. *The dry-weight concentration at harvesting, the lipid weight, the* P *content of the fractions and the Gram reaction of several micro-organisms*

Organism	mg.H/ 100 ml.	mg.L/ g.	μmole P/g. cell dry-weight							Gram reaction
			LP	AI	AO	RNA	DNA	XSP	Tot.	
Acetobacter pasteurianum (NCIB 8088)	30	.	48	15	57	472	53	−9	636	−
Aerobacter aerogenes (A)	210	49	62	50	46	407	.	4	594	−
Alcaligenes faecalis (NCTC 415)	150	85	87	27	37	388	97	13	649	−
Azotobacter agile (NCIB 8004)	20	121	148	25	72	.	.	.	701	−
Bacillus brevis (NCTC 7577)	70	48	45	31	29	410	73	−10	578	−
Escherichia coli (H)	100	68	63	26	42	345	180	8	664	−
Esch. coli (MS)	70	.	.	10	63	323	.	2	467	−
Proteus vulgaris (882)	120	70	73	48	22	365	94	3	625	−
Pseudomonas aeruginosa (NCTC 8203)	80	83	103	41	50	.	.	.	.	−
Serratia marcescens (NCTC 1377)	100	60	68	43	53	407	.	16	608	−
Neisseria catarrhalis (NCTC 3622)	70	81	82	26	163	560	123	−4	950	−
N. catarrhalis (NCTC 3622)	13	.	.	.	.	470	132	92	.	±
N. catarrhalis (NCTC 3622)	3	.	.	.	.	536	.	154	.	+
Baker's yeast (Distillers Co. Ltd.)	.	27	9·4	47	45	169	22	60	352	+
Bacillus subtilis (isolated from hay)	100	48	16	70	238	291	71	471	1157	+
Clostridium sporogenes (J)	40	51	42	71	56	426	75	103	773	+
Cl. welchii (SR 12)	30	55	45	37	30	491	130	226	959	+
Corynebacterium xerosis (NCTC 7247)	120	65	36	92	129	270	65	385	977	+
Lactobacillus casei (ATCC 7469)	25	63	28	63	129	188	84	168	660	+
Mycobacterium phlei (Söhn)	90	107	37	49	106	326	89	126	733	+
Staphylococcus aureus (Duncan)	300	60	38	166	110	197	73	250	834	+
Streptococcus faecalis (Dunn)	90	59	26	146	266	459	168	232	1297	+
Strep. faecalis (ST)	100	52	29	200	129	417	111	161	1047	+

negative group of organisms. The greatest difference is shown by the XSP fraction, which is present in all the Gram-positive organisms studied, but not present in significant amounts in the Gram-negative ones (Fig. 3). The total phosphate (Fig. 4) reflects the excess of AO, AI and XSP, and the relatively smaller deficit of LP in the Gram-positive group.

DISCUSSION

The present observations largely confirm our earlier results (Mitchell & Moyle 1950). Differences occur, however, because we previously used a less efficient method of lipid extraction with the result that part of the lipid phosphorus passed into the XSP fraction. We also employed a low mean mononucleotide extinction coefficient for nucleic acid (8200), taken from the literature, in place of the present experimentally determined value of 10,000 (Mitchell & Moyle, 1951*b*) in agreement with that of Wyatt (1950). The errors introduced by the above two factors were partially compensatory in most cases, but we were previously unable to demonstrate XSP in the clostridia. The present results show, however, that the clostridia, like all the other Gram-positive organisms studied, contain XSP.

There is a reciprocal relationship between XSP and LP content which suggests that the presence of the XSP-containing component may be related to the deficit of lipid phosphorus. For, whereas the mean P content of the lipid of the Gram-negative organisms (3·3% P) approximates to that of a phospholipid like lecithin (4·0% P), the mean P content of the lipid of the Gram-positive organisms is only about half as great (1·65% P).

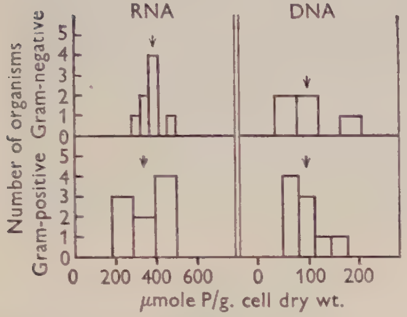

Fig. 1. The distribution of organisms with respect to RNA and DNA content.

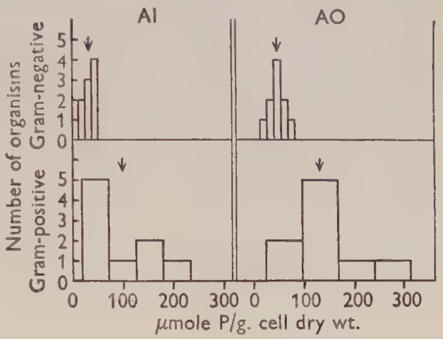

Fig. 2. The distribution of organisms with respect to AI and AO content.

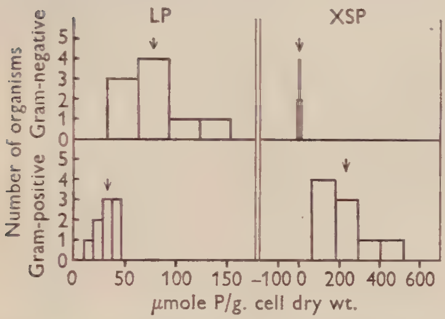

Fig. 3. The distribution of organisms with respect to LP and XSP content.

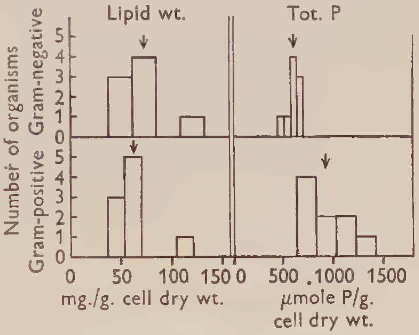

Fig. 4. The distribution of organisms with respect to lipid wt. and Tot. P content.

It should be borne in mind that the amount of lipid phosphorus obtained is dependent to a considerable extent on the method of extraction. The method which we have employed (repeated heating of the dry material with methanol and extraction with dry ether) was found by Reichert (1944) to yield an amount of lipid approximating closely to that obtained after mild acid hydrolysis. The method nevertheless remains an empirical one, the word lipid, in the present paper, being effectively defined by our method of extraction. The treatment with boiling methanol may be sufficient to break certain labile covalent bonds, releasing ether-soluble material which was originally chemically linked to insoluble components. On the other hand, it may be insufficient to release certain ether-soluble material which is attached to the residue only by van der Waals forces.

We included *Bacillus brevis* in the Gram-negative group of organisms because, in spite of its staining character, it possesses all the normal physiological characteristics of the Gram-positive aerobic spore-forming rods. The fact that *B. brevis* lacks XSP adds considerable weight to the correlation between XSP content and Gram staining, as does also the fact that 'young' Gram-positive cultures of *N. catarrhalis* contain XSP, whereas 'old' Gram-negative cultures do not. In *Staphylococcus aureus*, the XSP-containing component is a polyglycerophospho-compound, part of which is bound to the protein of the cell wall and part to the underlying lipo-protein membrane (Mitchell & Moyle, 1951*c*). We therefore suggest that a lipid-polyglycerophosphate-protein component may be a common structural feature of the cell envelopes of all Gram-positive bacteria, and that it is this feature which shows up as the XSP and low LP in the analyses of the whole cells.

The work of Newton (1953) and our own recent unpublished observations show that Gram-negative organisms lose significant amounts of the acid-soluble components when washed in distilled water, whereas Gram-positive organisms generally do not. Since all the organisms studied in the present work were washed with distilled water, the differences observed in the contents of the acid-soluble components may be due, at least in part, to a greater osmotic fragility of the envelopes of the Gram-negative group.

The values for the RNA and DNA contents of the organisms confirm our previous observations that the Gram-positive and Gram-negative groups of organisms do not differ significantly with respect to content of the nucleic acids (Mitchell & Moyle, 1950). The mean total nucleic acid phosphorus (RNA + DNA) content of all the organisms studied is *c.* 470 μmole/g., compared with an arginine content of *c.* 450 μmole/g. in Gram-positive organisms and *c.* 900 μmole/g. in Gram-negative ones. Thus, even without taking into consideration the XSP-containing component, the net negative charge attached to the cells (after fixation of the cells and loss of the small molecular weight solutes) must be much greater in Gram-positive than in Gram-negative organisms. This may explain the difference in the so-called isoelectric points of the Gram-positive and Gram-negative groups of bacteria (*c.* pH 3 and 5 respectively), measured by the pH at which the same amounts of an acidic and a basic dye are retained by the cells after staining in water and washing rapidly with ethanol (Stearn & Stearn, 1924). It has often been stated on the basis of the results of Stearn & Stearn that Gram-positive bacteria are more acidic than Gram-negative ones. We should note, however, that a large part of the difference in 'isoelectric point' should be attributed to the fact that Gram-positive bacteria are less basic than Gram-negative bacteria. On the other hand, Gram-positive bacteria may carry a greater number of negatively charged groups than Gram-negative bacteria because of the presence of the XSP-containing component. It is most unlikely that more than one hydroxyl group is free per phosphorus atom of the XSP-containing component, and hence the additional negative charge contributed by the XSP of the average

Gram-positive bacterium would not exceed *c.* 240 μequivalents/g.—somewhat more than half as great as the contribution due to the deficit of arginine (450 μmole/g.). The lower 'isoelectric point' of Gram-positive bacteria may therefore be attributed mainly to the presence of less basic groups.

When the movable ions are considered, the cells must, of course, be electrically neutral. It is therefore interesting that the deficit of the monovalent arginine residues in Gram-positive organisms (*c.* 450 μmole/g.) is largely compensated by the excess of the divalent magnesium ion (*c.* 190 μmole/g.). This suggests the following ion exchange hypothesis which has not, to our knowledge, been considered before as a factor in the Gram reaction. The uptake of crystal violet by the negatively charged components of Gram-positive cells may be accompanied by the displacement and escape of the magnesium counter ions, whereas in Gram-negative organisms the counter ions (being mainly the arginine residues of the cell proteins) may be displaced by the crystal violet but cannot escape. On washing with neutral ethanol, the stain is retained by Gram-positive cells because no counter ions are available, whereas it is not retained by Gram-negative cells because the arginine counter ions can move back to their original positions. We consider this hypothesis to merit consideration as an explanation for the ionic part of the mechanism of Gram staining which is probably predominant when the mordant is omitted. It may also explain the Gram staining reaction (including the use of a mordant) of extracellular components such as the magnesium protein nucleates of micro-organisms and artificial protein nucleates (Henry, Stacey & Teece, 1945) in which there is an excess of negatively charged groups associated with magnesium or other diffusible counter ions. The use of a mordant such as iodine, picric acid or mercuric iodide in the usual Gram method gives an incomparably sharper differentiation than can be obtained without a mordant (see, for example, Bartholomew & Mittwer, 1952); and it is probable that an additional mechanism to that of ion exchange must be responsible for the great increase in differentiation which is achieved. In view of the large decrease in intensity or complete loss of normal Gram staining which occurs when the cell envelopes are ruptured and the escaped cell contents are retained on the slide by fixation, it has often been considered possible that the cell envelopes themselves play a part in preventing the outward diffusion of the stain. This possibility deserves further study in view of the evidence for the difference of composition of the envelopes of Gram-positive and Gram-negative bacteria.

We are indebted to many of our colleagues in the Department of Biochemistry, Cambridge, for advice and criticism, in particular to Dr G. Weber and Dr D. H. Northcote. One of us (J.M.M.) wishes to acknowledge the receipt of a grant from the Medical Research Council in support of this work.

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(*Received* 16 *January* 1954)

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Seventeenth General Meeting in the University of Glasgow on Tuesday and Wednesday, 15 *and* 16 *September* 1953. *The following communications were made:*

COMMUNICATIONS

A Survey of 435 Strains of *Staphylococcus pyogenes*. By C. Lack (*Institute of Orthopaedics, University of London*)

Biochemical Tests for Proteus and Providence Cultures. By Patricia H. Clarke and Constance Shaw (*National Collection of Type Cultures, London*)

Only a few species of intestinal bacteria (*Proteus vulgaris*, *Pr. mirabilis*, *Pr. morganii* and *Pr. rettgeri*, the Providence group, including the Wakefield bacillus, Sachs's non-mannitol-fermenting dysentery organisms, and Stuart and Wheeler's anaerogenic paracolon organism type 29911) were able to produce phenyl pyruvic acid from phenylalanine by oxidative deamination. Tests were carried out in a salt base medium containing yeast extract and 1% phenylalanine, and also with washed suspensions in buffered phenylalanine. Shigellas, salmonellas, paracolons and coliforms were usually negative, although trace reactions occurred with some salmonellas and coliforms. This test can be used as a rapid diagnostic test for Proteus and Providence.

The Proteus and Providence cultures also produced leucine decarboxylase. A simple test was devised using washed suspension and amino-acid in phthalate buffer at pH 5·0 with bromcresol purple as indicator. No other enterobacteria tested attacked leucine, although under these conditions many cultures decarboxylated arginine and lysine. These results suggest that the Providence group and *Pr. rettgeri* are very closely related to the other species of the genus *Proteus*.

A Simple Selective Medium for the Primary Isolation of *Haemophilus pertussis* and *parapertussis*. By D. E. Nicholson and G. C. Turner (*School of Medicine, Leeds*)

Bordet-Gengou medium provides excellent conditions for the growth, not only of *Haemophilus pertussis*, but of many other organisms commonly found on per-nasal swabs. Incorporation of penicillin suppresses some of these, but others, including *H. influenzae*, remain unaffected.

Lacey (1951) devised a semi-synthetic medium which included 4 : 4-diamidino-diphenylamine dihydrochloride (M. & B. 938) at 12 μg./ml. and which was highly selective for *H. pertussis*. This has been tested over a period of 2 years with satisfactory results, but the medium is somewhat inhibitory to *H. pertussis*, and is unreliable for the isolation of *H. parapertussis*.

A simple selective medium, which not only allows good growth of *H. pertussis* and *H. parapertussis*, but also largely inhibits the growth of *H. influenzae*, can be made from horse meat extract and horse blood. Complete inhibition of *H. influenzae* and other organisms can be obtained by adding to the medium only 2 μg./ml. of the diamidine (which does not affect the growth of *H. pertussis* at this low concentration) and penicillin. The resulting medium is highly selective for *H. pertussis* and *H. parapertussis*.

500 g. lean horse meat (best steak) is simmered for 2 hr. with 1 l. distilled water containing 6 g. sodium chloride. The extract is filtered hot, and to the clear *fat-free* filtrate is added 10 g. Davis agar. The pH is adjusted to 7·6, and oxalated horse blood, penicillin and diamidine are added at 50° to give final concentrations of 20 %, 0·3 unit/ml., and 2 μg./ml. respectively.

So far, comparative trials show that with twenty-five positive swabs the new medium gave 26 % more positive isolations than on the classical Bordet-Gengou medium; and with just over fifty positive swabs 8·5 % more positive isolations than on Lacey's medium.

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Some Aspects of Bacteriological Standards for Foods. By J. G. Davis (*Reading*)

Specific Polysaccharides of Gram-negative Bacteria. By D. A. L. Davies (*Microbiological Research Department, Porton*)

Specific polysaccharides have been prepared by mild acetic acid hydrolysis from antigenic trichloracetic acid extracts of some Gram-negative bacteria. The nature of the sugar residues in these materials has been examined by chromatographic methods.

The polysaccharides prepared from *Shigella schmitzii* and *Sh. dispar* contained glucosamine, galactose, glucose and rhamnose. Specific polysaccharides from *Sh. dysenteriae*, *Sh. flexneri* and *Sh. sonnei*, which have already been described in the literature, contain glucosamine galactose rhamnose, glucosamine glucose rhamnose and glucosamine galactose glucose respectively; they thus contain glucosamine and the three possible combinations of two from the other three sugars galactose, glucose and rhamnose.

Examination of the polysaccharides extracted from eight Salmonella types having different 'O' antigenic constitutions showed that they all contained galactose, glucose, mannose and rhamnose though in different amounts; glucosamine was also present in all these materials but in very small quantities in some cases. In addition to these sugars the polysaccharides also contained one or two of the following: xylose, lyxose, an unidentified hexose, fucose and three unidentified deoxy-sugars. *Salmonella poona* specific polysaccharide contains chondrosamine and is serologically related to human blood group 'A' substance.

From eight strains of *Chromobacterium violaceum* polysaccharides were obtained which had little in common chemically, with the exception of two which both contained manno-gala-heptose, rhamnose and glucose; in each case the heptose composed the greater part of the material and glucose was present in small amount only. The polysaccharides from the other six strains contained from one to six of the following sugar residues: galactose, glucose, rhamnose, fucose, hexosamine, galacturonic acid, xylose, arabinose and an unidentified heptose.

Changes in the Bacterial Cell Wall during Cell Division as seen in the Electron Microscope. By I. M. DAWSON and H. STERN (*Glasgow University*)

The stages of bacterial cell division have been followed by electron microscope examinations of the isolated cell walls of *Staphylococcus aureus* and *Streptococcus faecalis*.

In both these organisms a transverse cell-wall septum is formed which originates in and grows centripetally from the cell wall. The septum splits into two layers and division is completed by constriction of the cell wall through the line of the double septum. Cell-wall septum formation and cell-wall constriction would appear to be dissociable phenomena.

Transverse cytoplasmic membrane septa, forming before the cell-wall septum, have been demonstrated in *Strept. faecalis.*

The Absorption of Polymyxin by Cell-wall Preparations from *Pseudomonas aeruginosa*. By B. A. NEWTON (*Medical Research Council Unit for Chemical Microbiology, University of Cambridge*)

Previous studies have shown that the addition of polymyxin to washed cell suspensions of *Ps. aeruginosa* caused a release of pentose, phosphate and materials which had an absorption maxima at 260 mμ. (Newton, 1953). No similar leakage was observed from washed cells of a resistant strain of *Ps. aeruginosa* which grew normally in 800 μg. polymyxin/ml.

Cell walls of a sensitive strain 'S' and a resistant strain 'R' were prepared by shaking washed cells, suspended in phosphate buffer, with small glass beads in a Mickle disintegrator. Cell walls of strain 'S' suspended in phosphate buffer and treated with polymyxin absorbed 330 μg. of the antibiotic/mg. dry weight of cell wall; this resulted in more than a 100 % increase in the turbidity of the suspensions. Walls of strain 'R' absorbed less than 70 μg. polymyxin/mg. dry weight cell wall and there was little increase in turbidity of these suspensions.

Electron microscopy has shown that polymyxin treatment of strain 'S' cell walls resulted in marked changes in appearance and the antibiotic could be seen adhering to them. The polymyxin was firmly bound to the walls and could not be removed by repeated washing or by further shaking with glass beads in the Mickle disintegrator. No change was observed in polymyxin treated cell walls of strain 'R'.

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The Effect of Arsenate upon the Bacterial Breakdown of Citrulline.

By V. A. Knivett (*Medical Research Council Unit for Chemical Microbiology, University of Cambridge and Medical Research Council Unit for Research in Cell Metabolism, University of Sheffield*)

The breakdown of citrulline by cell-free extracts of *Streptococcus faecalis* in the presence of magnesium ions, and adenosine diphosphate (ADP) is stimulated by either phosphate or arsenate, whereas both together are inhibitory. In the presence of arsenate the breakdown of citrulline is competitively inhibited by phosphate and no ADP is required. Fluoride and *p*-chloromercury benzoate are effective inhibitors. The rate of release of CO_2 from citrulline is dependent upon the concentrations of arsenate and citrulline. The disappearance of citrulline is accompanied by the formation of equimolar quantities of ornithine, NH_3 and CO_2.

Accelerating effects of arsenate in enzymic reactions (reviewed by Crane & Lipmann, 1953) are taken to be due to the formation of an arsenate analogue of a phosphorylated intermediate which differs from the phosphate derivative in being readily hydrolysed. The cell-free extracts were subjected to a purification procedure, and various fractions were tested for activity in promoting citrulline breakdown (*a*) in the presence of phosphate and ADP, and (*b*) in the presence of arsenate. Maximum activity in (*a*) did not coincide with maximum activity in (*b*), and the relative activity *b*/*a* varied from 0·9 to 60. It is concluded that two enzymes are involved. In the presence of citrulline, phosphate and a 'phosphorylase', a phosphate derivative of citrulline is formed. The phosphate is transferred to ADP through the mediation of a 'phosphokinase'. The action of arsenate is attributed to the formation of an unstable arsenate derivative of citrulline by the 'phosphorylase' (*b*).

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Some Studies on a Pyrimidine-requiring Mutant of *Aerobacter aerogenes*.

By R. H. Nimmo-Smith (*University of Oxford*)

The Tricarboxylic Acid Cycle in *Bacterium coli*.

By K. McQuillen (*Department of Biochemistry, University of Cambridge*)

Growing cultures of *Bacterium coli* strain B convert various ^{14}C-labelled substrates into cellular material. Experiments with $^{14}CO_2$ (Abelson, Bolton & Aldous, 1952) and with $^{14}CH_3COOH$ and $CH_3{}^{14}COOH$ (McQuillen & Roberts, 1954) can be interpreted in terms of the tricarboxylic acid (TCA) cycle.

A theoretical treatment yields flow diagrams for the various parts of the cycle, and predicts qualitatively and quantitatively the positions and activities of labelling in various products after administration of a given tracer. The flow patterns established by the use of one tracer can be checked by using others; results obtained with $^{14}CO_2$, $^{14}CH_3COOH$ and $CH_3{}^{14}COOH$ are all mutually consistent when the same experimental conditions obtain.

During exponential growth at 37° C., 30–35 % of the oxalacetate formed as an intermediate comes from α-ketoglutarate, while the rest is formed by CO_2/pyruvate condensation. Since oxalacetate and α-ketoglutarate (or the corresponding amino-acids) are precursors of some eight amino-acids, it appears that more than half of the protein carbon of *B. coli* passes through some part of the TCA cycle during the synthesis of amino-acids. It is further possible to calculate that only a small fraction (*c.* 1/6) of the glucose carbon metabolized during growth enters the cycle, and that an even smaller fraction of the CO_2 production is due to reactions of the cycle.

These findings imply that the TCA cycle in growing *B. coli* is of fundamental importance in furnishing intermediates for amino-acid synthesis, but has a minor role in carbohydrate oxidation.

This work was carried out at the Carnegie Institution of Washington in collaboration with Dr R. B. Roberts.

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The Action of Nucleic Acids on the Incorporation of Glutamic Acid by Fragmented *Staphylococcus aureus*. By E. F. Gale (*Medical Research Council Unit for Chemical Microbiology, University of Cambridge*)

^{14}C-labelled glutamic acid is incorporated into the protein fraction of disrupted staphylococcal cells if these are incubated with adenosinetriphosphate, hexosediphosphate and the labelled amino-acid in the presence or absence of a complete mixture of other amino-acids. When other amino-acids are missing, the incorporation appears to take place as a result of an exchange between the added glutamic acid and glutamyl residues of the cell protein (Gale & Folkes, 1953; Gale, 1953). If the disrupted cells are first extracted with M-NaCl, the incorporation process is markedly increased by the addition of nucleic acid preparations worked up from the soluble portion of the cells. The nucleic acid preparation is partially inactivated by incubation with either ribonuclease or desoxyribonuclease. Ribonucleic and desoxyribonucleic acid have now been prepared from the whole staphylococcal nucleic acid, and both preparations are found to activate glutamic acid incorporation when this occurs under exchange conditions, DNA being approximately twice as effective as RNA compared on a dry weight basis.

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Some Properties of an Oxalate-decomposing Bacterium. By G. C. N. Jayasuriya (*A.R.C. Unit for Microbiology, University of Sheffield*)

A bacterium utilizing oxalate as carbon source has been isolated from soil after enrichment at 25° in a medium containing 1 % (w/v) potassium oxalate and inorganic salts. The organism differs from other known oxalate decomposers by forming no red pigment (cf. *Bacillus extorquens* (Bassalik, 1913)) and by failing to grow on acetate or formate (cf. *Vibrio oxaliticus* (Bhat & Barker, 1948); *Pseudomonas oxaliticus* (Janota, 1951)).

The organism grows in concentrations of potassium oxalate up to 2 % (w/v) (optimum 0·8–1·0 %) and at pH from 6·5 to 9·0 (optimum 7·0–8·0). Growth is preceded by a lag of about 18 hr. which cannot be reduced by the addition of yeast autolysate, bicarbonate or formate. Of several other substrates tested only glycolate, DL-lactate, pyruvate, DL-malate and succinate support growth.

Washed cells oxidize oxalate, and the gas exchanges observed correspond with an assimilation of 10 % of the oxalate used. Succinate, fumarate, malate, pyruvate, lactate, acetate, glycolate and formate are also oxidized. The oxidizing powers of washed cells vary with the growth substrate, e.g. lactate-grown cells oxidize lactate and glycolate more rapidly and oxalate less rapidly than oxalate grown cells.

In the presence of 0·001M-monofluoracetate the inhibition of the rate of oxidation of glycolate and oxalate (58 and 55 %) is much greater than with acetate or pyruvate as substrate (6 and 8 %). Under these conditions glycolate gives rise to more than twice the amount of citrate obtained with pyruvate. Oxalate and acetate give no citrate. This appears to exclude the possibility that glycolate is an intermediate in the oxidation of acetate by this organism.

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DEMONSTRATIONS

G. Pontecorvo, J. A. Roper, R. A. Pritchard and C. E. Forbes (*University of Glasgow*). ‘Techniques for the genetics of filamentous fungi (*Aspergillus nidulans, A. niger* and *Penicillium chrysogenum*).’

I. M. Dawson and H. Stern (*University of Glasgow*). ‘Micrographs of changes in the bacterial cell wall during cell division.’

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